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Characterisation of a haemolysin produced by *Vibrio splendidus*, a pathogen of turbot larvae.

Hazel Louise Macpherson

**Presented for the degree of Doctor of Philosophy in the Division of Infection and
Immunity, Institute of Biomedical and Life Sciences, Faculty of Science, The
University of Glasgow**

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Immunohistochemistry was carried out at the Institute of Marine Research, Bergen with the help of Dr Øivind Bergh. Nucleotide sequence determination was carried out by the Sequencing Service, University of Durham.

Hazel Macpherson

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Abbreviations

bp	Base pair
cfu	Colony forming Units
cm	centimetre
d	Day
dH ₂ O	Distilled water
ECP	Extracellular products
EDTA	Ethylenediamine tetra acetic acid
EMBL	European Molecular Biology Laboratory
FHL	Filtered haemolymph
g	Gram
h	Hour
H ₃ PO ₄	Phosphoric acid
HTH	Helix-turn-helix
HU	Haemolytic Unit
kb	Kilobase
kD	Kilodalton
L	Litre
LAB	Lactic acid bacteria
LB	Luria Bertani
M	Molar
mA	Milliamps
MA	Marine Agar
MB	Marine Broth
MgSO ₄	Magnesium sulphate
min	Minute
ml	Millilitre
ml ⁻¹	per millilitre
mm	Millimetre
mM	Millimolar
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NCIMB	National Collection of Industrial and Marine Bacteria

ng	Nanogram
nm	Nanometre
nmoles	Nanomoles
(NH ₄) ₂ SO ₄	Ammonium sulphate
OD	Optical Density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylenic glycol
pfu	Plaque forming units
pmol	Picomol
rpm	revolutions per minute
SDS	Sodium dodecyl sulphate
sec	Second
SSW	Sterile seawater
spp.	Species
Taq	<i>Thermus aquaticus</i>
TCBS	Thiosulphate Citrate Bile Salts Sucrose
TE Buffer	Tris-EDTA buffer
TSA	Tryptone soy agar
TSB	Tryptone soy broth
µg	Microgram
µl	Microlitre
µm	Micrometre
UV	Ultraviolet
V	Volts
v/v	Volume for volume
w/v	Weight for volume
w/w	Weight for weight
λ	Lambda
‰	Parts per thousand

Summary

Although turbot is produced in substantial quantities in aquaculture in Europe the major limitation in expansion of the industry is the supply of juvenile fish for on-growing. High mortalities can occur during early life stages during the critical times of transition from yolk-sac larvae to feeding on rotifers and *Artemia*. There is good evidence that microorganisms are the major cause of the problems associated with early-feeding, as improved survival is seen when antibiotics are used or when larvae are reared in bacteria-free conditions. Several studies have shown that the bacterial flora of the larval turbot gut originates from the food. However, high levels of certain bacteria in the gut flora are not harmful, rather it is particular bacterial types that appear to determine larval rearing success.

The present study concentrated on determining the virulence mechanisms of *Vibrio splendidus* DMC-1 (biovar 1), which was isolated from a batch of turbot larvae suffering very high mortality at a turbot hatchery. This isolate was shown to be lethal in turbot larvae challenge assays with immunohistochemistry studies of challenged larvae showing signs of enteritis in the gut.

A protein of c. 40 kDa with an N-terminal amino acid sequence showing homology with the OmpU outer membrane protein from *V. vulnificus*, but other potential virulence factors secreted by this organism were investigated. *Vibrio splendidus* DMC-1 grown in Marine Broth produced high titres of haemolysin and haemagglutinin against turbot blood. The haemagglutinin appeared specific for turbot cells and may function as an adhesin.

The haemolysin had a broad spectrum of cell-damaging activity and was partially purified and shown to be a heat-labile pore-forming protein toxin, producing membrane channels of approximately 1.8 nm diameter. The toxin was secreted into the culture medium during logarithmic growth with production being subject to catabolite repression, with medium composition governing the level of haemolysin produced.

Transposon mutagenesis was used to identify the haemolysin gene. Over 10,000 mutants were screened, resulting in isolation of 3 fully haemolysin-negative (hly-)

mutants and several with reduced expression of haemolysin. Two different hly-mutants that were non-haemolytic, non-cytotoxic to turbot tissue culture cells and avirulent against turbot larvae were characterised. Over 8 kb of the *V. splendidus* genome was sequenced to identify the haemolysin gene and neighbouring genes. Overall, the DNA sequence had high homology with sections of the genomes of *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*, with the same genes and gene order as in these species. In addition, *Vibrio splendidus* contained a section of DNA inserted into this common *Vibrio* background that was unrelated to the above vibrios but that closely resembled the gene for aerolysin, a pore-forming cytotoxin from the *Aeromonas* genus that has not previously been recognised outside that genus. The predicted molecular weight of this toxin was 55 kD. The presence of this toxin could explain the enteritis found in the larval turbot gut as the haemolysin-negative transposon mutant lacked the ability to cause cell damage *in vitro* and *in vivo*. In the second haemolysin-negative mutant the transposon insertion site was in a gene adjacent to that for the cytotoxin. The translation product of this gene showed homology with a well-established group of regulators of bacterial virulence gene expression, known as ToxR. Analysis of the secondary structure of this regulator indicated that it possesses all the domains necessary for gene regulation but with novel features not previously recorded for other regulators of virulence genes. As transposon mutagenesis of the ToxR-like gene causes loss of haemolytic activity it suggests that this gene is functional and controls expression of the haemolysin, but it is not known whether other genes are also regulated by this system.

Chapter 1 Introduction

1.1. Background

Aquaculture is one of the fastest-growing food-producing sectors in the world. In 2001 the total fishery production in the world was 130.2 million tonnes, of which 37.9 million tonnes were from aquacultural practices (FAO, 2004). The majority of fish produced from aquaculture is from S.E. Asia with China being the largest producer. Marine aquaculture has rapidly expanded in Europe mainly due to the culture of Atlantic salmon in Norway, Scotland and Ireland with over 500,000 tonnes now produced per annum (FAO, 2004). In fact, aquaculture in Europe has increased steadily from 10 % of total fish production in 1989 to 15 % in 1998 (FAO, 2002).

Aquaculture has a history dating back several centuries to China, when carp was raised in rice paddies. Research into fish biology and the development of farming techniques has provided the tools for farming on a commercial basis. There is continued demand for high value fish species, and with catches from wild capture fisheries reaching a maximum exploitation level, aquaculture is expected to meet the increasing demand for fish.

New species suitable for large-scale culture have been sought to allow the industry to diversify and expand its production. These species included turbot (*Scophthalmus maximus*), halibut (*Hippoglossus hippoglossus*), cod (*Gadus morhua*), sea bream (*Sparus aurata*) and sea bass (*Dicentrarchus labrax*). These species were chosen for aquacultural rearing due to their ease of growth and high market value as well as to meet demands due to stocks dwindling in the wild.

1.2. History

Turbot belongs to the family *Scophthalmidae* and is widely found in the Atlantic Ocean and along European coasts. The intensive rearing of turbot is well established in Europe especially in Northern Spain (Riaza and Hall, 1993), where over 5000 tonnes are produced per annum (Riaza, personal communication). However the turbot farming industry is faced with expansion limitations, as are other marine fish species, in that there are difficulties in producing sufficient quantities of larvae and juveniles (Riaza and Hall, 1993; Shields, 1999; 2001). High mortalities (up to 90 %) can occur

during the "critical period" of rearing, when larvae transform from the yolk-sac, endogenous feeding to the exogenous first feeding stage of development (Munro *et al.*, 1994; Gibson and Johnstone, 1995; Blanch *et al.*, 1997; Hansen and Olafsen, 1999; Ringø and Birkbeck, 1999). The successful rearing of turbot depends on the hatchery outputs and predicting this depends on the growth and survival of larvae which is a problem due to the high variation experienced between batches of larvae.

1.3. Turbot rearing

In the wild, turbot larvae hatch in coastal waters of less than 40 metres in depth and larvae between 3.5 and 6.0 mm feed on copepod nauplii, whereas larvae measuring up to 12 mm feed on *Temora* copepodites and cladocerans *Podon* and *Evadne* (Reay, 1979). In aquaculture, larvae are first fed on live feed of rotifers for a duration of 8 to 10 days, and are then offered the brine shrimp *Artemia* until day 30-40. The larvae can then be weaned onto an artificial inert diet. It is known that there are severe nutritional deficiencies in this diet, and one way in which this has been improved is through the enrichment of rotifers and *Artemia* with unicellular algae such as *Isochrysis* and *Pavlova* (Paulsen, 1989). Also, the *Artemia* can be enriched with oils to provide essential nutrients for the fish (Gatesoupe, 1991a; Sorgeloos *et al.*, 2001).

Wild turbot naturally spawn between April and August. In aquaculture it is possible to produce eggs all year round through the inducement of spawning. Altering the length of daylight through manipulation of photoperiods enables this, so that turbot broodstock can be induced to produce eggs throughout the entire year (Paulsen, 1989). The eggs can be stripped from the females and artificially fertilised. The stripping of the females occurs over a period of 3-6 weeks, and should be performed less than 10 hours after ovulation, as eggs can become overripe. The fertilised eggs are incubated to enable hatching, the larvae are reared beyond metamorphosis and transferred to on-growing facilities (Reay, 1979; Riaza, personal communication). The larvae and juveniles require an optimum temperature of 17°C for growth but this high temperature is not required for larger fish, therefore, allowing on-growing sites to be more manageable.

1.4. Rearing Conditions

Important factors that influence the success of larviculture include the water quality, light intensity and dilution rate of live food or algae (Planas and Cunha, 1999). The use of algae for 'green water' and dark-walled tanks has been shown to produce larvae with improved appetite, growth rate, survival and viability (Skjermo and Vadstein, 1993; Reitan *et al.*, 1997). In addition to these effects the algae also appeared to improve the tank and larval gut microflora (Nicolas *et al.*, 1989; Reitan *et al.*, 1997).

1.5. Development of the gut of turbot

The development of the digestive tract is an important factor to consider in the formation of the gut microflora (Ringø and Birkbeck, 1999). In fish, the digestive tract begins as a simple, straight non-differentiated tube adjacent to the yolk-sac, which is unlike the complex system in adult fish. In early development, the gut lengthens, twists and develops pyloric caecae (pouches) (Segner *et al.*, 1994). The length increase slows down at metamorphosis but the mucosal surface area continues to increase. No functional stomach is present during the first-feeding stage as the stomach only becomes functional at metamorphosis of the turbot. Therefore, the food is mainly digested in the intestine which decreases the capability of efficient digestion of exogenous protein, resulting in a demand for food rich in amino acids and delaying the weaning onto a formulated diet (Segner *et al.*, 1994). However, studies on winter flounder (*Pleuronectes americanus*) have discovered that the stomach develops at around day 20 post-hatch, a week before weaning onto a formulated diet (Douglas *et al.*, 1999). Therefore, earlier weaning may be advantageous, as it would shorten the time of the problematic live feed administration.

The larvae have the intestinal mucosal barrier to protect them from the complex microbial populations that make up the intestinal microflora. The important factors that influence the gut microflora of mammals, such as gastric acid, bile salts and proteolytic enzymes are present in fish larvae, but little information is known of their role in the natural defence system. It is expected that the destruction of ingested bacteria by acid in the stomach and bile in the intestine does not occur in the larval system because the gut has not completely developed at such an early stage. As a result the larvae are more prone to bacterial infections (Segner *et al.*, 1994).

1.6. Gut colonisation

As a result of the ongoing development of the intestinal tract during first feeding through to metamorphosis, the associated microorganisms undergo a two-step pattern of change from a heterogeneous unstable flora into a stable homogeneous flora (Bergh, 1995; Ringø *et al.*, 1996; Ringø and Vadstein, 1998). There are few, if any, bacteria associated with the digestive tract at the time of hatching of turbot larvae (Munro *et al.*, 1993; 1994; Ringø *et al.*, 1996; Blanch *et al.*, 1997; Ringø and Vadstein, 1998) or other flatfish larvae (Muroga *et al.*, 1987; Tanasomwang and Muroga, 1988; Skjermo and Vadstein, 1993). In contrast to this, the wolffish *Anarhichas lupus* L. fry are morphologically well developed at the time of hatching and have a distinct intestinal gut flora immediately after hatching (Ringø *et al.*, 1987).

Bacterial colonisation of fish larvae is complex and depends on the flora of:

1. eggs
2. larval rearing water
3. live food

The early life stages of the fish larvae in intensive rearing systems take place in incubators in the presence of both hatching eggs and debris, therefore, it was thought likely that the egg microflora would be an important factor in the establishment of indigenous larval microflora (Bergh, 1995; Keskin *et al.*, 1994). It has been demonstrated that newly hatched yolk-sac larvae of cod (*Gadus morhua* L.) ingest substantial amounts of bacteria and older larvae graze on the egg debris (Hansen and Olafsen, 1989).

Bacterial adhesion and colonisation of the egg surface occurs within hours after fertilisation, with the composition and amount reflecting that of the ambient water (Hansen and Olafsen, 1999). The development of the egg into the yolk-sac stage involves the ingestion of bacteria from the surrounding environment which can constitute the primary intestinal microflora. These initial colonisers can persist beyond first feeding (Hansen and Olafsen, 1999). Non-specific mechanisms act to defend the egg and the larvae as the immune system is immature at this stage.

In natural populations of turbot larvae the microflora of the gut might reflect that of the aquatic environment, however, no such studies have been reported. In artificial rearing environments the flora is altered by the use of matured water, microalgae, live food and antibacterials.

The drinking rate of larvae increases from 14 to 120 nl per individual per hour from day 2 to 11 after hatching in order to osmoregulate (Reitan *et al.*, 1998). During this time they may ingest a small number of algae, bacterial particles and egg debris (Beveridge *et al.*, 1989). Larvae must also take in water to ventilate gills and particles may become trapped during this process at rates considerably higher than those of drinking. During the first day after hatching the digestive system is poorly developed so colonisation of the gut is a relatively non-selective process. A study by Ringø *et al.* (1996) concluded that drinking activity before first feeding resulted in the colonisation of the gut with *V. pelagius* in turbot larvae soon after hatching. Also, Ringø and Vadstein (1998) found that the bacterial species *V. pelagius* and *Aeromonas caviae* colonised the gut of larvae when added to water at day 2 post-hatch.

Live feed in aquaculture is widely used, the most common at first feeding of turbot is the rotifer, *Brachionus plicatilis* or *Brachionus rotundiformis* followed by the brine shrimp *Artemia*. Studies have shown that once first feeding commences the intestinal flora is similar to the live feed microflora rather than that of the surrounding water. (Muroga *et al.*, 1987; Tanasomwang and Muroga, 1988; Munro *et al.*, 1993; 1994; 1999). Thus, during first-feeding when relatively high amounts of rotifers are consumed, a stable protective flora may develop to protect the gut from harmful bacteria, but at the same time this provides an opportunity for pathogens to infect the larvae. The change in microflora at this stage is qualitative and quantitative with an increase in the bacterial numbers and a shift from non-fermentative species associated with the eggs, such as *Cytophaga* and *Flexibacter* species, to fermentative *Aeromonas* and *Vibrio* species (Munro *et al.*, 1994; Bergh *et al.*, 1994; Blanch *et al.*, 1997). Normally, there are few bacteria present in gut of newly hatched turbot larvae, however after first feeding commences there is a rapid increase with greater than 10^4 colony forming units (cfu) per fish recovered on day 4 (Munro *et al.*, 1993). After days 4-5 post-hatch the number of bacteria per fish did not greatly increase.

A positive correlation was observed between *Vibrio* levels and types found in *Artemia* to those found in feeding turbot larvae (Gatesoupe, 1990; Olsen *et al.*, 2000). *Vibrio alginolyticus* dominates the microflora of *Artemia* cultures and has been found in the feeding turbot larvae (Blanch *et al.*, 1997), summer flounder (*Paralichthys dentatus*) larvae (Eddy and Jones, 2002), olive flounder (Tanasomwang and Muroga, 1988), sea bream and sea bass larvae (Grisez *et al.*, 1996). This bacterial species was shown to disappear when larvae were weaned onto dry formulated feeds.

The majority of bacteria associated with rotifers may be externally located, with the total aerobic population ranging from 10^7 to 10^{10} cfu per gram of dried rotifers (Munro *et al.*, 1993). Perez-Benavente and Gatesoupe (1988) concluded that high numbers of bacteria associated with rotifers and *Artemia* were detrimental to the turbot larvae as disinfection with antibiotics 24 hours prior to feeding improved larval survival. Munro *et al.* (1999) also found that the reduction of the bacteria on the rotifer surface using UV treatment increased the survival of the turbot larvae. It has been shown that a slow rate of gut colonisation produced improved survival rates (Munro *et al.*, 1993), which may explain the results of the disinfected live food trials.

Artemia can also harbour bacteria on their surface that can be detrimental to the feeding larvae with isolates including *Erwinia*, *Bacillus*, *Micrococcus*, *Staphylococcus* and *Vibrio* spp. The bacteria surrounding the *Artemia* nauplii can easily be removed by using decapsulated cysts that have been sterilised with hypochlorite to reduce the amount of bacteria entering the rearing system (Austin and Allen, 1981; Sorgeloos *et al.*, 2001).

1.7. Larval Microflora

The development of a stable microflora is widely accepted as being important in the maintenance of human and animal health. As with higher vertebrates the intestinal microflora of fish must adapt to the varying conditions of nutrient composition, pH, anaerobiosis, concentration of bile salts and digestive enzymes, the host's immune system and the mutual influences of other members of the intestinal bacterial

community. The bacterial flora of fish has to be well adapted to cope with the fluctuations between the nutrient rich intestinal tract and the comparatively oligotrophic ambient seawater.

The population level of bacteria in fish intestines is lower and simpler than that of warm-blooded animals (Ringø *et al.*, 2001). However it is accepted that fish possess a specific intestinal microflora consisting of aerobic, facultative anaerobic and obligate anaerobic bacteria, of which the composition may change with age, nutritional status and environmental conditions (Hansen and Olafsen, 1999). The existence of such an intestinal microflora in larvae has led to the assumption that some microorganisms are autochthonous, while others are regarded as abnormal and may lead to disease.

To identify autochthonous microorganisms in the gastrointestinal tract of fish the bacteria must:

1. Be found in healthy individuals
2. Colonise early life stages and persist throughout life
3. Be found in both free living and hatchery cultured fish
4. Grow anaerobically
5. Be found associated with the epithelial mucosa in the stomach and large intestine.

The methods employed to identify microorganisms from the gut of larvae and live food involves rinsing surface of larval fish with 0.1% benzalkonium chloride saline solution to remove surface bacteria (Muroga *et al.*, 1987; Munro *et al.*, 1994; Blanch *et al.*, 1997). Larvae or live feed are then homogenised and isolates selectively grown and characterised. However, from the homogenised sections of the gut not all organisms grow, thus, biasing the population, as some of the strains can go undetected (Ringø *et al.*, 2001).

The bacterial flora associated with the flatfish species of skate (*Raja* species) and lemon sole (*Pleuronectes microcephalus*) was studied by Liston (1957) and that of plaice (*Pleuronectes platessa*) by Gilmour *et al.* (1976). The main species identified

from the skin and gills were *Pseudomonas*, *Achromobacter*, *Alcaligenes*, *Flavobacterium*, and *Vibrio* species. However the gut microflora was distinct and consisted mainly of "gut group vibrios" (Liston, 1957).

The intestinal microflora of larval and juvenile stages of farmed red sea bream (*Pagrus major*) and black sea bream (*Acanthopagrus schlegeli*) was studied by Muroga *et al.* (1987). The bacterial levels in the intestine were 7.4×10^4 and 3.4×10^4 colony forming units (cfu) per fish, for red and black sea bream, respectively. The genera *Vibrio* and *Pseudomonas* were dominant, accounting for 45 % and 30 % of the total culturable bacteria, respectively. Tanasomwang and Muroga (1988) revealed similar strains in the gut microflora of larval and juvenile Japanese flounder (*Paralichthys olivaceous*). The *Pseudomonas* species were found to be predominant in the water and the live food. However, the detection of vibrios was much lower, thus suggesting that gut colonisation is selective. Campbell and Buswell (1983) carried out a similar study on the intestinal microflora of farmed Dover sole (*Solea solea*). The initial dominant bacterial groups were *Pseudomonas* and *Alcaligenes* that corresponded to the flora in the tank water. As the larvae increased in age there was an increase in the levels of *Vibrio* and *Aeromonas* that corresponded to the flora of the live food.

A study by Bergh (1995) on halibut larvae showed that there was a shift in the intestinal flora from the onset of feeding. The flora changed from *Cytophaga*, *Flexibacter* and *Flavobacterium* species to *Vibrio* and *Aeromonas* species (Bergh *et al.*, 1994; Verner-Jeffreys *et al.*, 2003a). The transition in the gut flora occurred when the larvae began feeding on live food, resulting in a fermentative flora dominated by *Vibrio* and *Aeromonas* species (Bergh *et al.*, 1994; Verner-Jeffreys *et al.*, 2003a). The reason for this shift in inhabiting microflora may have been the influx of food alongside diversification of the gut. At first, the population is very heterogeneous with an increase in a diverse range of *Vibrio* spp. that later become homogeneous. In fact, in a study by Cerdà-Cuellar and Blanch (2004) the microflora of turbot larvae at the end of development was homogeneous belonging mainly to a single phenotype of *V. scophthalmi*.

Munro *et al.* (1994) showed the same bacterial colonisation in a study of the gut microflora of turbot (*Scophthalmus maximus*). The gut colonisation coincided with the start of feeding with live food and was dominated with *Vibrio* and *Aeromonas* species. In a further study by Munro *et al.* (1993), *V. alginolyticus* was found to be the dominant *Vibrio* species of the turbot gut. Studies on gut colonisation by Blanch *et al.* (1997) found *V. splendidus* to be the most abundant *Vibrio* associated with the gut of rotifer feeding larvae. Both of these organisms were found in the gut of the feeding halibut larvae by Verner-Jeffreys *et al.* (2003a). On the whole a high proportion of the bacterial isolates identified in the larval gut after first-feeding were haemolytic (Angulo *et al.*, 1994a).

The genus *Vibrio* comprises more than 35 species, which are widely distributed in the marine environment. Vibrios are the most common species in the gut of fish (Muroga *et al.*, 1987; Bergh, 1995; Munro *et al.*, 1994). For example, Munro *et al.* (1994) found that *Vibrio* species accounted for >70% of the gut isolates, with *V. alginolyticus*, *V. pelagius*, *V. cambellii*, and unidentified vibrios frequently being isolated. Some of these organisms can be pathogenic specifically if the host is under stress.

Vibrio species are not dominant in either the water or the food in aquaculture therefore it may be that they possess specific characteristics that could favour their implantation into the gut (Blanch *et al.*, 1997). From studies on the gut microflora it was concluded that the indigenous microflora of fish intestines arose from diet and became selectively established due to the specific conditions in the gut. As suggested by Ringø and Birkbeck (1999) the production of enzymes such as proteases, amylase, chitinase and lecithinase may account for their favourable colonisation of the gut.

The intestinal tract of turbot at larval and fry stages has been examined in an attempt to characterise the bacterial species present (Table 1.1).

Fish Species	Bacterial genera	References
Turbot, <i>Scophthalmus maximus</i>	Vibrionaceae <i>Vibrio alginolyticus</i> , <i>Aeromonas</i> <i>V. alginolyticus</i> , <i>V. natrigens</i> , <i>V.</i> <i>anguillarum</i> , <i>V. fluvialis</i> , <i>V.</i> <i>pelagius</i> , <i>Aeromonas caviae</i> , <i>Acinetobacter</i> , <i>V. alginolyticus</i> , <i>V. anguillarum</i> , <i>V.</i> <i>campbellii</i> , <i>V. fluvialis</i> , <i>V. furnissi</i> , <i>V. harveyi</i> , <i>V. natrigens</i> , <i>V. nereis</i> , <i>V. ordalii</i> , <i>V. pelagius</i> , <i>V.</i> <i>splendidus</i> , <i>Vibrio</i> , <i>Aeromonas</i> , <i>Pseudomonas/Alcaligenes</i> , <i>Flavobacterium/Cytophaga</i> , Enterobacteriaceae, <i>Acinetobacter</i> , <i>Photobacterium</i> , <i>Moraxella</i> <i>Aeromonas</i> , <i>Vibrio</i> , Enterobacteriaceae, <i>Cytophaga</i> , <i>Micrococcus</i> , <i>Staphylococcus</i> , coryneforms Oxidative Gram-negative rods, <i>V.</i> <i>natrigens</i> , <i>V. pelagius</i> , <i>V.</i> <i>scophthalmi</i> , <i>V. splendidus</i> , <i>V.</i> <i>mediterranei</i> , <i>V. anguillarum</i> , <i>V.</i> <i>proteolyticus</i> , <i>V. alginolyticus</i> <i>Acinetobacter</i> , <i>Moraxella</i> , <i>Vibrio</i>	Nicolas <i>et al.</i> 1989 Gatesoupe, 1990 Munro <i>et al.</i> 1993 Munro <i>et al.</i> 1994 Ringø <i>et al.</i> 1996 Blanch <i>et al.</i> 1997 Gatesoupe <i>et al.</i> 1997

Table 1.1 Overview of microflora identified from turbot larval gut (modified from Ringø and Birkbeck, 1999).

Lactic acid bacteria (LAB) within the gut of endothermic animals have been extensively studied and reviewed and have been found to be the dominant members of the microflora of the early life stages (Goldin and Gorbach, 1992; Fuller, 1989). They are a group of gram-positive organisms that include members of the genera *Streptococcus*, *Enterococcus*, *Lactobacillus*, *Aerococcus*, *Carnobacterium*, *Leuconostoc*, *Lactococcus*, and *Pediococcus* (Ringø and Gatesoupe, 1998). Few investigators have isolated LAB from the gastrointestinal tract of larval and juvenile marine fish (Ringø and Gatesoupe, 1998). This may be due to the prolonged incubation times required and the absence of glucose in the selective media used. *Lactobacillus* and *Carnobacterium* have been shown to colonise the gut of fish (Baya

et al., 1991; Ringø and Gatesoupe, 1998; Ringø, 1999; Ringø and Holzapfel, 2000; Ringø *et al.*, 2000).

1.8. Bacterial pathogens identified in larval rearing.

The relationship between microorganisms and larvae is likely to be complex and a range of factors will influence these interactions. At such an early developmental stage, the larvae possess reduced immunocompetence thus making them more susceptible to disease (Ellis, 1989). As the larvae develop they will become less fragile and therefore more likely to be resistant to invasion by opportunistic bacteria.

The high survival attainable with larval turbot in the absence of bacteria shows the role they have in influencing larval mortality (Munro *et al.*, 1995). However, it was also demonstrated that the turbot larvae could develop normally in the presence of high concentrations of bacteria. Thus it is probable that particular bacterial species play a major role in determining the survival of larval fish in ways that remain to be recognised. Therefore, it is more important to control the bacterial types rather than the density in the larval system.

Under intensive rearing conditions, a stable microbial community is difficult to achieve as sudden increases in nutrients occur when exogenous food is added (Skjermo and Vadstein, 1999). The combination of high larval densities, debris from dead larvae, high loads of organic matter and bacteria added with the intensively produced live food, favours the growth of opportunistic bacteria. Several studies with larval turbot (Nicolas *et al.*, 1989; Munro *et al.*, 1993; Munro *et al.*, 1994; Blanch *et al.*, 1997) failed to reveal the presence of recognised bacterial pathogens and opportunistic pathogens are more likely to be the cause of disease in larvae (Munro *et al.*, 1994; 1995; Verner-Jeffreys *et al.*, 2003b). Nevertheless, pathogens have been associated with some cases of high mortalities among larval fish (Muroga *et al.*, 1990). *Vibrio splendidus* were isolated from moribund larval turbot and certain isolates have been shown to be pathogenic to turbot larvae (Gatesoupe *et al.*, 1999). This organism was originally identified as an *Aeromonas* species (Gatesoupe, 1990; 1991b) but later shown to be a *Vibrio* sp. (Gatesoupe, 1995). Not all *V. splendidus* isolated from the turbot rearing systems were virulent (Gatesoupe *et al.*, 1999) but the

reasons for this were not established. *Vibrio splendidus* strains were also isolated from dying turbot larvae feeding on *Artemia* from larval rearing systems in Spain and these were shown to be pathogenic in larvae (Thomson, 2001). Similar *V. splendidus* organisms were identified from the *Artemia* production systems in halibut hatcheries (Verner-Jeffreys, 2000; Verner-Jeffreys *et al.*, 2003a) although these have not been shown to be harmful to larvae. As a result it was presumed this was the probable route of entry of this organism into larval systems. However seawater has also been recognised as a reservoir for this organism (Lopez and Angulo, 1995).

Bacterial colonisation of eggs occurs rapidly in seawater, and studies by Hansen and Olafsen (1989) and Hansen *et al.* (1992b) have shown the eggs of cod and halibut to be heavily contaminated with bacteria. Bergh *et al.* (1992) clearly demonstrated that the dominant bacteria (*Flexibacter* sp. now known as *Tenacibacter*) present on halibut eggs influenced larval survival and were pathogenic for halibut eggs. Studies on bacterial colonisation of turbot eggs have been carried out by Keskin and Rosenthal (1994). Unviable eggs were covered with bacterial biofilms by day 3 of incubation and the presence of these dead eggs was thought to be responsible for the colonisation of the viable eggs. The studies found that 3.7×10^6 bacteria per viable egg were present on day 4 of incubation. However, no known pathogens have been isolated from the egg stage of turbot.

1.8.1. Vibriosis

Vibriosis is the most common type of bacterial disease of marine species and is the most important in cultured marine species (Plumb, 1999). In Norwegian experience with cultured turbot, vibriosis can occur in the very early life stages (Egidius, 1987). A range of *Vibrio* species have been reported to cause disease in fish including *V. alginolyticus*, *V. anguillarum* (*Listonella anguillarum*), *V. carchariae*, *V. cholerae*, *V. damsela*, *V. harveyi*, *V. ordalii*, *V. parahaemolyticus*, *V. mimicus*, *V. vulnificus*, and *V. salmonicida* (Plumb, 1999). Austin and Austin (1999) also reported the above strains and in addition to these, *V. marinus* (*Moritella marina*), *V. fisheri*, *V. furnissii*, *V. ichthyenteri*, *V. logei*, *V. pelagius*, *V. splendidus*, *V. trachuri*, and *V. viscosus* (*M. viscosa*) were also reported to cause disease in fish. The strains best noted for their

pathogenicity in fish are *V. anguillarum*, *V. salmonicida*, *V. vulnificus* biotype 2 and *V. ordalii* (Toranzo *et al.*, 1997; Plumb, 1999).

It is well known that vibrios are disease agents in adult and juvenile turbot. However, individual species are seldom identified as a major constituent of the larval microflora. However, the fish larvae contain an intestinal microflora dominated by *Vibrio* species as a result of the intake of live food that is heavily contaminated with *Vibrio* species, therefore, posing a potential risk to the larvae if an opportunist strain proliferates.

1.8.1.1. *Vibrio anguillarum*

Vibrio anguillarum has been described as a primary pathogen for several farmed species, including gilt-head seabream (*Sparus aurata*) (Balebona *et al.*, 1998) and turbot (Horne *et al.*, 1977; Horne and Baxendale, 1983). The intestine of turbot has been proposed as the portal of entry for *V. anguillarum* (Olsson *et al.*, 1996; 1998), but the mode of infection is unclear from the literature. It was suggested that *V. anguillarum* invaded through the skin, the gills or the anus (Laurecin and Germon, 1987), but in contrast, Muroga *et al.* (1990) and Grisez *et al.* (1996) suggested the route of entry of *Vibrio* species was via the food chain. The *V. anguillarum* bacteria were endocytosed in the intestine and from there entered into the blood stream to cause eventual disease (Grisez *et al.*, 1996). Therefore, if the live food is contaminated with an enteric pathogen like *V. anguillarum*, enteric vibriosis can occur (Grisez *et al.*, 1996). *Vibrio anguillarum* has been isolated from diseased turbot and sea bass (*Dicentrarchus labrax*) in Norwegian fish farms (Myhr *et al.*, 1991). In experimental infections with *V. anguillarum* (isolated by Horne *et al.*, 1977), high mortalities occurred in turbot larvae (Munro *et al.*, 1995) and in halibut larvae (Verner-Jeffreys *et al.*, 2003a). Another *V. anguillarum* strain (HI-11360) isolated from Norwegian turbot was pathogenic towards turbot larvae (Skiftesvik and Bergh, 1993). Episodes of high mortalities in farmed sea bream and sea bass larvae were found to be associated with high numbers of *V. anguillarum* (Grisez *et al.*, 1997). Therefore, as for juvenile and adult marine fish, the presence of *V. anguillarum* in larval rearing systems can be considered a major threat.

1.8.1.2. *Vibrio splendidus*

Gatesoupe (1997) found isolates of *Vibrio splendidus* isolated from turbot to be pathogenic in turbot larval trials (Gatesoupe, 1997). However non-pathogenic *V. splendidus* isolates were also detected (Gatesoupe *et al.*, 1999). Variability between isolates does occur due to heterogeneity, which has been demonstrated before with *V. splendidus* isolates in fish (Austin *et al.*, 1997).

Vibrio splendidus has been isolated from several disease outbreaks of farmed adult turbot fish (Myhr *et al.*, 1991; Lupiani *et al.*, 1993). A disease outbreak in North West Spain in farmed turbot caused by a protozoan, saw the surviving fish suffer from further disease due to an outbreak of vibriosis (Angulo *et al.*, 1994b). The bacterium isolated was identified as *V. splendidus* biotype I, and was positive in virulence assays of rainbow trout (*Oncorhynchus mykiss*) and turbot. In addition to this isolation, acute mortalities in juvenile New Zealand hatchery reared turbot, *Colistium nudipinnis* suffering from vibriosis symptoms had *V. splendidus* I and *V. campbellii*-like variants isolated from liver, kidney and spleen. However, an epizootic study of a turbot farm in Spain detected *V. splendidus* in 20-75 % of healthy fish (Novoa *et al.*, 1992), further evidence that supports other work (Balebona *et al.*, 1998), that such pathogens in rearing systems may be opportunistic.

1.8.1.3. *Vibrio pelagius*

In turbot larvae a *V. pelagius* strain was isolated as the cause of mass mortalities at a turbot farm in Spain (Villamil *et al.*, 2003). However in studies by Ringø *et al.* (1996) and Ringø and Vadstein (1998) a *V. pelagius* strain isolated from turbot larvae in the U.K. was applied to turbot larvae as a potential probiotic, and in the latter study was shown to decrease mortalities caused by *A. caviae*. *Vibrio pelagius* was also detected in 20-75 % of healthy turbot fish from a farm in Galicia, Spain (Novoa *et al.*, 1992) showing that it is widely distributed in turbot culture systems.

1.8.1.4. Other vibrios

Other species identified as primary pathogens in fish include *V. alginolyticus* and *V. harveyi*, the latter strain only being identified as an opportunistic pathogen (Balebona *et al.*, 1998). *Vibrio alginolyticus* caused low level mortalities in aquarium-reared

turbot suffering from gill disease (Austin *et al.*, 1993), suggesting that this is an opportunistic bacterium that can invade stressed fish or damaged tissue. In salmonids, *V. harveyi* has been found to be pathogenic causing luminous vibriosis (Zhang and Austin, 2000) but is better recognised as a pathogen of the tiger prawn, *Penaeus monodon* (Liu *et al.*, 1996).

1.8.1.5. Other pathogens

The absence of reports in the literature makes it unclear whether turbot larvae are at risk to pathogens of cold water fish like *Renibacterium salmoninarum*, *M. viscosa*, *Piscirickettsia salmonis* and *Vibrio salmonicida*. As mentioned above, it is probable that the main risk of turbot larvae is not from recognised pathogens as rarely have pathogenic bacteria been isolated from rearing systems or has a correlation been made between the presence of particular bacterial isolates and mortalities (Munro *et al.*, 1993; 1995; Tanasomwang and Muroga, 1988).

Aeromonas salmonicida subspecies *salmonicida* is the causative agent of furunculosis in salmonids and increasingly in other fish species (Bernoth, 1997). Atypical *A. salmonicida* was found in turbot from salt-water tanks in Denmark (Pedersen, 1994). However, infection trials with Atlantic halibut larvae found they were only mildly susceptible to challenge with *A. salmonicida* subsp. *salmonicida* (Bergh *et al.*, 1997).

Enteric red mouth (ERM) is a systemic bacterial disease of trout caused by *Yersinia ruckeri*, also known as Hagermann red-mouth disease, redmouth salmonid blood spot and 'yersiniosis' (Plumb, 1999). Rainbow trout is the species most severely affected by ERM, however all salmonids are susceptible and non-salmonids like turbot and sturgeon can be infected but mortalities have been insignificant.

Bacteria, viruses and protozoa have all been implicated as pathogens in flatfish culture. Birnaviruses and IPN-like viruses have been isolated from turbot however they were not causing mortalities (Novoa *et al.*, 1992). There was an outbreak of viral haemorrhagic septicaemia (VHS) on the island of Gigha in Scotland in 1994 (Ross *et al.*, 1994). VHS was traditionally considered to be a disease of rainbow trout

(*Oncorhynchus mykiss*), where extensive losses have occurred in freshwater operations across continental Europe. Castric and de Kinkelin (1984) were the first to report that turbot were susceptible to VHSV, following experimental infection. The virus isolated from the outbreak on Gigha was found to be similar to many VHSV isolations from several species of wild marine fish from the north east Atlantic and the North Sea (King *et al.*, 2001). Therefore, it could be speculated that the virus originated in wild fish.

1.8.2. Pathogenesis of *Vibrio* infections.

The general external disease symptoms in adult fish are haemorrhages and superficial skin lesions, and in most cases general septicaemia (Egidius, 1987). Other symptoms include abdominal distention, focal haemorrhagic lesions in the kidney and the liver, loss of appetite, discoloration of the skin and congestion of the fins (Horne *et al.*, 1983). Even although the diseases caused by *Vibrio* species have been known and studied extensively for nearly a century, little is known about their pathogenesis and pathology.

Pathogenic species produce various virulence factors including enterotoxin, haemolysin, cytotoxin, protease, siderophore, adhesive factor or haemagglutinin, which are proteinaceous except for the siderophore (Shinoda, 1999).

Bacterial adherence to host cells is often an essential step to initiate infection because it enables localisation of the pathogens to the appropriate target tissues in many infections (Finlay and Cossart, 1997). Adherence is mediated by adhesins that can be funbrial (fimbriae or pili) or afimbrial (outer membrane proteins, OMP; lipopolysaccharide LPS). Salinity, pH and cell surface hyrophobicity (Krovacek *et al.*, 1987) also affect adhesion. Adhesion to the host cells or mucus in fish may result in the internalisation via endocytosis or phagocytosis, or it may allow the pathogens to cause localised damage if they excrete toxic components (Wang *et al.*, 1998).

Fish have a mucus layer covering primary and secondary gill lamellae, the skin and the gastrointestinal tract, all of which may act as adhesion sites or function to prevent firm attachment of bacteria (Hansen and Olafsen, 1999; Knudsen *et al.*, 1999). Mucus is the first line of defence containing factors like immunoglobulins, complement,

lysozyme and agglutinins, and continual shedding and subsequent renewal of mucus deters microbes from attachment.

The adhesion processes and mechanisms for various mammals have been studied in detail, however, few reports exist on adhesion of bacteria to mucosal surfaces of fish. Knudsen *et al.* (1999) reported that *V. salmonicida*, *V. viscosus* (*M. viscosa*), *Flexibacter* (*Tenacibacter*) *maritimus*, and *V. anguillarum* serotype O1 all adhered *in situ* to mucus on salmon mucosal surfaces (i.e. gills, skin, pyloric caeca, foregut and hindgut). *Vibrio anguillarum* serotype O2 adhered to other tissue components and not mucus; thus, it may possess a mechanism for invasion (Knudsen *et al.*, 1999). Krovacek *et al.* (1987) investigated adhesion of *A. hydrophila* and *V. anguillarum* to fish cells and postulated that adhesins are involved in this mechanism. Adhesion of autochthonous bacteria of turbot to intestinal mucus of fish was assayed *in vitro* by Olsson *et al.* (1992) who found them to adhere strongly to the intestinal mucus, compared to skin bacteria that were poorly adhesive.

Receptor-specific interactions are common in mammals; for example colonisation of the small intestine by enterotoxigenic *Escherichia coli* is mediated by pilus-like adhesins. Other organisms that bind to host epithelia through sugar residues to establish an infection are *Salmonella typhi* and *V. cholerae*. According to Hansen and Olafsen (1999) LPS, especially the carbohydrate moiety of the molecule, is involved in adhesion of marine vibrios as for mammalian pathogens. There is great variation in the carbohydrate moiety of LPS, which may explain the variation in adhesiveness of *Vibrio* strains.

Santos *et al.* (1991) investigated the cell-surface associated properties of fish pathogens. The majority of *A. salmonicida*, motile *Aeromonas* and *V. anguillarum* were moderately hydrophobic, whereas *Y. ruckeri* had weak or very low hydrophobicity. Thus the cell surface hydrophobicity may not be important to the pathogen's adhesion abilities. However, Conway *et al.* (1986) found that there was increased hydrophobicity in the gut flora of starved or stressed fish.

1.8.2.1. Pathogenesis of *Vibrio anguillarum* infections

Several virulence factors are known for the fish pathogen *V. anguillarum* that enable it to survive and multiply in non-immune fish. A highly effective iron-sequestering system, (Wolf and Crosa, 1986; Crosa, 2001), an extracellular zinc metalloprotease (Norqvist *et al.*, 1990) and the ability to resist the bactericidal effect of fish serum have been described (Trust *et al.*, 1981). In addition, *V. anguillarum* produces an extracellular heat-stable toxin, a heat-labile exotoxin and a haemolytic toxin which are presumed to act together in infection (Farrell and Crosa, 1991). All of these virulence attributes enable the pathogen to survive and proliferate in the blood eventually leading to septicaemia and death.

The presence of metalloproteases in the microbial world has been suggested as a strategy for survival but they are not necessarily associated with virulence. In a study of 288 marine isolates, 60% showed caseinase activity and 77% produced gelatinase activity (Simidu and Tsukamoto, 1985). Extracellular proteases are distributed in *Vibrio* spp. irrespective of their pathogenicity and origin (Brown and Roland, 1984). Nottage and Birkbeck (1987) found *Vibrio* spp. lacking protease were still pathogenic to shellfish larvae.

However, extracellular proteases are well known for their roles as virulence determinants in some bacteria. Norqvist *et al.* (1990) detected a zinc metalloprotease that was associated with the invasion process. The zinc-metalloprotease secreted by *V. anguillarum* shares properties with those of *V. vulnificus* and *V. cholerae* (Hase and Finkelstein, 1993) and there is usually extensive homology in the N-terminal sequence of metalloproteases from different vibrios.

In addition to the proteases, other extracellular products (ECP) in *V. anguillarum* have been studied and it was demonstrated that haemolysins were involved in virulence (Munn, 1978; Choe and Jeong, 1995). Haemolysins are the most widely distributed toxins in pathogenic vibrios (Iida and Honda, 1997). Munn found the haemolysins were heat-labile factors, with activity being detected after 19 hours growth that peaked at 39 hours and then declined. Inactive haemolysins were also secreted that were later activated. Munn (1978) postulated two explanations for the limited detection of

haemolysin, suggesting that it was only produced during stationary phase or that production occurred intracellularly followed by release into the environment through autolysis of the bacterial cells.

Production of haemolysin can also be involved in the acquisition of iron from haemoglobin (Fouz *et al.*, 1993). Increased levels of haemolysin were detected in the supernatants of iron-starved cells of *V. cholerae* (Stoebner and Payne, 1988). The haemolysins of *V. anguillarum* and *V. vulnificus* have been found to lyse erythrocytes to release heme and haemoglobin which proteases can then act upon to liberate a source of iron from the host for the bacterial cell to utilise (Mazoy and Lemos, 1991; Fouz *et al.*, 1996).

Therefore, toxin production in *Vibrio* spp. can play a major part in infections. However, a study that examined the haemolytic activity, adhesiveness to cell surfaces and cytotoxic activity to fish cell lines found no correlation between virulence and the cytotoxicity of ECP with both the virulent and non-virulent strains being haemolytic (Toranzo *et al.*, 1983).

Another virulence mechanism reported in *V. anguillarum* strains isolated from fish is the ability to haemagglutinate red blood cells (Trust *et al.*, 1981; Toranzo *et al.*, 1983). It was suggested that haemagglutinating activity is closely related to adhesion and plays an important role in the infectivity of the pathogen. However, contrary to the findings by Trust *et al.* (1981) there was no correlation between virulence and haemagglutination of fish erythrocytes (Toranzo *et al.*, 1983).

The exact mode of action of *V. anguillarum* is unclear, but it is obvious that it does involve colonisation, which is then succeeded by penetration. Chemotactic motility in *V. anguillarum* was found to be essential for virulence (Milton *et al.*, 1996; O'Toole *et al.*, 1996). O'Toole *et al.* (1999) showed that *V. anguillarum* and *V. cholerae* possess a chemotactic motility towards the mucus of their respective hosts, however, it is not host specific. This chemotactic motility is not dependent on one chemoattractant as the organism can respond to a range of chemoattractants in intestine/stomach mucus. Therefore, this is a virulence mechanism that enable *V. anguillarum* to colonise different epithelial surfaces and fish species. It was

postulated that the flagellin A protein aids invasion and plays a role in virulence once the fish integument has been crossed by the pathogen (Milton *et al.*, 1996). The penetration of the mucus layer in the intestine was also postulated by Olsson *et al.* (1996) to involve chemotaxis rather than adhesion mechanisms. However, Ormonde *et al.* (2000) found that motility rather than individual components of the flagellum is required for invasion of the fish host. In studies with *Campylobacter jejuni* motility was found to be required for the invasion of intestinal cells and flagellin A served as a secondary adhesin to the intestinal cells (Yao *et al.*, 1994). In addition to this, the flagellum of *V. cholerae* has been implicated in pathogenicity as a motility or adhesin component to aid invasion (Richardson, 1991).

Therefore, colonisation would explain why *V. anguillarum* was able to survive in the stomach of turbot, proliferate in the intestine and still be detected in the faeces (Olsson *et al.*, 1998). The adhesion to intestinal sections was also reported by Horne and Baxendale (1983). A study by Ringø *et al.* (2001) found bacterial cells were endocytosed in the pyloric caeca and midgut of adult fish. In larvae, the endocytosis of bacteria by enterocytes occurred mainly in the epithelial border of the foregut. This study clearly indicated that the intestine is involved in bacterial endocytosis (Ringø *et al.*, 2001). With *V. anguillarum* infection it was proposed that the intestinal cells endocytosed the bacteria (Grisez *et al.*, 1996).

The main virulence factor of *V. anguillarum* was postulated to be the presence of the pJM1 plasmid contained within serogroup O1 strains (Crosa, 2001). This virulence plasmid encoded an iron-sequestration system that allowed the bacterial cell to compete for available iron in fish tissues. The iron-sequestering system has also been reported in strains of *V. damsela* (Fouz *et al.*, 1994; 1997). However, Toranzo *et al.* (1983) detected virulent strains that did not contain the plasmid and were still able to grow in iron-limited conditions. The strains that were able to grow in the iron-limited conditions were found to produce an additional outer membrane protein (OMP) and possess a separate iron-uptake system present on the chromosomal DNA (Lemos *et al.*, 1988). Mackie and Birkbeck (1992) have demonstrated the siderophore production by *V. anguillarum* in infected fish. Nevertheless, iron-uptake systems have been detected in non-pathogenic strains indicating that other factors are also important in the pathogenesis of *V. anguillarum*.

The production of specific OMPs by *V. anguillarum* is thought to be necessary for the survival and protection of the strain that allows it to adapt to the different environments of marine water and the intestinal microflora of fish. The OMPs protect the bacterial cell contents from any damaging substances whilst allowing the uptake of nutrients (Achouak *et al.*, 2001). One such protein was detected by Davey *et al.* (1998) and identified as a 40 kD OMP that was homologous with OmpU of *V. cholerae* and OmpC of *E. coli*. A 40 kD porin was also characterised by Simón *et al.* (1996). This was regarded as a common antigen in *Vibrio* spp. (Simón *et al.*, 1998).

A further study by Wang *et al.* (2003) identified a 38 kD OMP with 75 % homology with *V. cholerae* OmpU. It was postulated that this OMP played a role in bile resistance but no reduction in virulence was seen on deletion of the gene for this protein and bile resistance was probably due to the presence of a second OMP (37 kD). Therefore, the 37 kD OMP may be preferentially expressed in the intestinal environment, similar to the relationship between OmpC and OmpF in *E. coli*. OmpC is expressed in the high osmolarity and high temperature conditions of the human body, whereas, OmpF is expressed in the low osmolarity, low temperature conditions of the environment. The 38 kD OMP of *V. anguillarum* was postulated to be important in limiting biofilm formation outside the fish host which aids in the adaptation to environmental changes and thus pathogenic capabilities (Wang *et al.*, 2003).

The number and nature of the virulence factors involved in the pathogenesis of vibriosis are still unclear. In a study of *V. anguillarum* and *A. hydrophila* both the pathogenic and non-pathogenic strains showed similar enzymatic and toxic profiles, with the ECP displaying amylolytic, proteolytic, haemolytic, cytotoxic and dermatotoxic activities (Santos *et al.*, 1992). Therefore, virulence is multifactorial with a combination of virulence factors rather than one specific mechanism being required to cause disease.

1.8.2.2. Pathogenesis of other *Vibrio* species

In *V. damsela* (*Photobacterium damsela*) infections ECP have been implicated as virulence factors. The ECP produced by this organism had low proteolytic activity,

no caseinase, elastinase or gelatinase activity (Fouz *et al.*, 1993) but did possess haemolytic and phospholipase activity against turbot, human and sheep erythrocytes, and cytotoxicity towards fish cell lines. These results were in agreement with those of Kothary and Kregger (1985) who observed a correlation between disease and the production of a toxin, damselysin that was haemolytic and cytolytic. Another virulence mechanism that contributes to *V. damsela* pathogenicity and is common to other vibrios was the presence of a siderophore-mediated iron-sequestering system (Fouz *et al.*, 1997).

Vibrio splendidus and *V. alginolyticus* are known to produce extracellular proteases, esterases, haemolysins and aggressins likely to assist in invasion and colonisation (Hase and Finkelstein, 1993; Diggles *et al.*, 2000).

ECP have also been identified in the pathogenesis of *V. harveyi*, with haemolytic activity being high in virulent strains (Liu *et al.*, 1996; Zhang and Austin, 2000). However, in a study by Zhang and Austin (2000) it was found that other factors were contributing to the overall pathogenicity as well as ECPs as there was high variation between *V. harveyi* strains.

As mentioned for *V. anguillarum*, the mechanism of fish vibriosis is multifactorial and the production of varying amounts of different virulence factors by different strains of *Vibrio* spp. is common. For example in *Escherichia coli* infections the pathogen must possess at least two virulence factors before being able to cause disease in a susceptible host. It first must be able to colonise the gut epithelium using adhesins and then produce an enterotoxin to cause the diarrhoea (Sussman, 1997).

1.9. Possible Control Strategies

1.9.1. Vaccination

The use of vaccines now enables control of a range of bacterial fish pathogens and long-term protection against *V. anguillarum* can be induced using vaccines of killed bacterial cells (Toranzo *et al.*, 1997). However, difficulties still remain in the development of effective vaccines against certain bacteria and viruses. Vaccination against vibriosis is successful in adult turbot fish (Dec *et al.*, 1990) but it is not feasible in the yolk-sac and first-feeding larval stages. Vaccination has proved

difficult at the larval stage of teleost fish because their immune system is immature early in their development (Ellis, 1989; Low *et al.*, 2001). Therefore exposure to a vaccine early in development may result in poor responses when faced with challenge at a later stage. This is characteristic of the response in mammals where the early exposure to an antigen results in the suppression of later immune responses. Therefore, the larvae are not amenable to vaccine application due to their immature immune system and their small size.

1.9.2. Immunostimulants

An alternative method of disease control is the application of immunostimulants like glucans and vitamins (Sakai, 1999). Immunostimulation of juvenile turbot has been carried out using alginate incorporated into live food (Skjermo *et al.*, 1995). Again, however, the immaturity of the immune system may affect the effectiveness of these strategies.

1.9.3. Alternative methods

1.9.3.1. Control of bacteria in system

There are various means by which the larval rearing systems can be controlled and manipulated in order to influence the bacterial flora that develops in these systems. Vadstein *et al.* (1993) considered three approaches, firstly to reduce bacterial levels non-selectively, secondly to enhance particular groups of bacteria selectively and thirdly to improve or enhance the larval resistance to bacteria. One of these methods alone was not expected to be successful, rather a combination of various techniques will be required to improve the overall production in the larval rearing system.

1.9.3.2. Non-selective reduction of bacteria

Non-selective reduction of bacteria can be achieved by the use of disinfectants, antibiotics and other means of sterilisation like ozone and UV treatment. The surface disinfection of eggs is routinely employed in salmonid aquaculture to reduce the probability of disease transfer from parent to offspring and between hatcheries (Bergh, 1995). The most suitable disinfectant is glutaraldehyde, which gives good bactericidal effects with no adverse effects on eggs or larvae. Antibiotics are commonly used in

laboratory trials to remove contaminating bacteria from surfaces but if used in the fish farm this can lead to the emergence of resistant strains. It is important to avoid prolonged exposure of eggs to antibiotics because this can be damaging to larval growth, development and survival (Olafsen, 2001).

As described by Keskin *et al.* (1994) the viable eggs of turbot become colonised with bacteria as a result of incubation in the same tanks as the non-viable eggs contaminated with thick bacterial biofilms. Therefore, even with the use of UV-sterilisation of water and surface sterilisation of the eggs (Evelyn *et al.*, 1984) the problem cannot be resolved. The best solution would be to reduce the density of eggs in the incubation tanks and regularly remove unviable eggs (Keskin *et al.*, 1994).

As discussed earlier the microflora of the larvae is affected by the addition of live food to the rearing systems. This is also the stage where the majority of mortalities occur, and potentially where pathogens enter the system (Campbell and Buswell, 1983; Muroga *et al.*, 1987; Nicolas *et al.*, 1989). It has already been noted that there are high levels of bacteria associated with the live food of rotifers and *Artemia* (Muroga *et al.*, 1987; Nicolas *et al.*, 1989; Tanasomwang and Muroga, 1988; Munro *et al.*, 1994; Verschuere *et al.*, 1997; 1999; Olsen *et al.*, 1999; 2000; Eddy and Jones, 2002). Munro *et al.* (1995) found that feeding bacteria-free rotifers increased the survival rates of turbot larvae. Also, turbot fry fed with *Artemia*, reared under axenic conditions by the application of antibiotics to the rearing system, showed better survival and growth rates compared to fry that had been fed normal *Artemia* (Perez-Benavente and Gatesoupe, 1988).

The total aerobic bacterial population of rotifers ranges from 10^7 to 10^{10} colony forming units per gram of dried rotifers (Munro *et al.*, 1993). It was suggested by Keskin *et al.* (1994) that bacteria from the water or on the surface of the rotifers and *Artemia* should not be a problem if rotifers and *Artemia* are well rinsed prior to feeding to the larvae. However, this does not stop the ingested bacteria of the live food entering the larvae.

Munro *et al.* (1993) provided evidence that most of the bacterial load of *Brachionus plicatilis* was located on the exterior surface of the rotifer. Several methods to reduce the bacterial load have been tried, the most promising of these methods was ultraviolet (UV) irradiation. Disinfecting live food by UV-irradiation reduced the bacterial load by >99% with no adverse effects (Munro *et al.*, 1993). In a further study the use of UV radiation was highly effective at reducing the bacterial load of rotifers by 90 % (Munro *et al.*, 1999). Although the bacterial numbers were reduced successfully, re-growth occurred within 48-hours, but the number of bacteria per UV-treated rotifer was still only approximately 40% of that of untreated rotifers (Munro *et al.*, 1999). In the same study, attempts were made to colonise the turbot larval gut with selected bacterial strains using both normal and UV-treated rotifers, but with no success.

In other studies very high larval survival was achieved in a bacteria-free rearing system that also allowed colonisation of larvae with defined bacteria (Munro *et al.*, 1995; Douillet, 1998). Therefore, these studies proved that the reduction or elimination of the bacterial load on the live food could be achieved. However, it would be impractical to use these methods in a large-scale rearing system.

The *Artemia* exist for long periods of time in a dormant cyst form and may contain contaminating bacteria. The cysts are decapsulated in hypochlorite or dilute sodium hydroxide solutions, to completely disinfect (Puentes *et al.*, 1992; Sorgeloos *et al.*, 2001). However, they are very quickly recolonised with bacteria during the breaking stage shortly before hatching. At this stage glycerol is released from the cysts offering an ideal culture medium for the growth of *Vibrio* species (Sorgeloos *et al.*, 2001). The *Artemia* are cultivated in intensive conditions and are fed fish meal and fish oil to enrich them with nutrients for the larvae (Olsen *et al.*, 2000). Under such conditions opportunistic bacteria can readily proliferate (Verschuere *et al.*, 1997). Many bacteria associated with *Artemia* have been identified as *Vibrio* species, which have been identified as potential opportunistic pathogens of turbot larvae (Nicolas *et al.*, 1989), and high numbers of haemolytic *Vibrio* spp. were found in *Artemia* used to feed Atlantic halibut larvae (Olsen *et al.*, 2000).

Generally, there are no attempts to control the microbial flora of larval hatcheries except for the treatment of incoming water by filtration and UV-irradiation, with occasional resort to the use of antibiotics. Therefore, additional strategies such as the application of defined bacteria to prevent opportunistic pathogens from colonising the gut would be extremely useful developments for fish farms. Challenge experiments have shown that larvae are susceptible to disease caused by pathogens incorporated into the live food of rotifers and *Artemia* (Masumura *et al.*, 1989; Munro *et al.*, 1995; Grisez *et al.*, 1996) and methods are required to reduce or eliminate the introduction of pathogens via the live food organisms.

It has been demonstrated that the preemptive colonisation with selected bacterial strains could influence the bacterial microflora and possibly prevent the proliferation of pathogens and opportunists in the cultures of *Artemia* (Verschuere *et al.*, 1999; 2000a; Makridis *et al.*, 2000a and b). The addition of selected bacterial strains dramatically influenced the composition of the bacterial microflora of the live food in two separate studies (Verschuere *et al.*, 2000a; Makridis *et al.*, 2000a). A different approach was taken by Olsen *et al.*, (2000) who succeeded in changing the bacterial numbers and composition by the addition of *Tetraselmis* sp. microalgae to give a more diverse flora in *Artemia* cultures. The algae probably altered the substrates in the gut of the *Artemia* that favoured the growth of opportunistic bacteria. Similarly, in rotifer cultures the addition of lactic acid bacteria (LAB) reduced the concentration of aerobic bacteria and increased the growth rate of the feeding turbot larvae (Gatesoupe, 1991a).

1.9.3.3. Selective enhancement of bacteria

Antibiotics are frequently used during the hatching and the early larval life stages to reduce bacterial numbers (Hansen and Olafsen, 1989). However, the problem of drug resistance among fish pathogens has increased steadily (Håstein, 1995) and there has been progressive selection of resistant strains. Therefore, the beneficial effects of antibiotics may be eliminated and instead of a decrease, there is an increase in disease incidence (Håstein, 1995). The use of antibiotics and chemicals in aquaculture has been restricted in recent years because of the above reason (Björklund *et al.*, 1990; Austin and Al-Zahrani, 1988). Antibiotics have been shown to persist in the

sediments at fish farms and this could have effects on both the farmed and wild fish as well as human health (Samuelsen, 1989; Björklund *et al.*, 1990). Bacterial strains can survive antibiotic treatment because they carry genes for resistance. These strains, whether pathogenic or not, can grow rapidly because their competitors are removed. It is then possible that these strains can pass their genetic information on to other potential pathogens to allow the evolution of other resistant strains.

Aeromonas salmonicida, that causes furunculosis in salmonid fish, has been found to develop resistance to quinolones, oxolinic acid and flumequine due to a chromosomal mutation (Nielsen *et al.*, 1994). As well as chromosomal genes, transferable resistance genes carried by plasmids (R-plasmids) can be responsible for the development of resistance (Aoki *et al.*, 1984). This plasmid activity is an important issue, as transfer may occur between the aquatic bacteria to the taxa of human and/or animals. Aoki *et al.* (1981) detected these transferable drug resistance R-plasmids in *V. anguillarum* strains isolated from diseased ayu in Japan. The high incidence of R-factors in bacteria from cultured ayu was assumed to be due to the selective pressure exerted by chemotherapeutic agents used in aquaculture, as no resistant bacteria were isolated from wild ayu.

As a result, alternative approaches to controlling the bacteria in larval rearing systems have been sought. One such application is the use of 'probiotics', which involves the use of beneficial bacterial species to manipulate the gut microflora and reduce the effects of potentially harmful species. The concept of biological disease control in aquaculture using non-pathogenic bacterial strains has received widespread attention (Ringø and Birkbeck, 1999; Ringø and Gatesoupe, 1998; Gatesoupe, 1999; Gram and Ringø, 2004).

1.9.4. Probiotics

The definition by Fuller (1989) of a probiotic being "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal balance" is commonly used. However, a better definition was given by Gram *et al.* (1999) as "a live microbial supplement which beneficially affects the host animal by improving its microbial balance". The latter definition is more applicable to an aqueous

environment where the application of microbes to the food or the water could alter the microbial balance in a beneficial way.

The use of probiotics as feed supplements for humans and farm animals has been practised effectively for about 30 years to promote a beneficial gut microflora (Fuller, 1999). The products are of varying composition and efficacy but the concept is scientifically based and intellectually sound. Lactic acid bacteria are one example of potential probiotics as they occupy a central role in the gut flora of all endothermic animals, they are easy to culture in large quantities and are considered 'safe' because of their long history of use in food fermentation (Berg, 1998).

Hume *et al.* (1998) selected probiotic bacteria to protect chickens from salmonella by using a continuous culture approach, to mimic the conditions in the chicken digestive tract, resulting in colonisation. The mixture (PREEMPT) was shown to be successful in large-scale commercial use and represents an excellent mode for developing a true 'normal' gut flora, that protects against pathogens. Another example is a milk replacement inoculated with *Lactobacillus* and *Streptococcus* cultures, given to young calves that resulted in decreased faecal coliform counts and a reduction in the occurrence of scouring and an overall improvement in their growth (Fuller, 1999).

According to Atlas (1999), for probiotics to be used, it is necessary to have detailed knowledge of:

- The pathogen against which protection is sought, its mechanisms of virulence, proliferation and invasion.
- The host, its immune defence, and its natural microflora.
- The surrounding environment, including nutrients, microorganisms, etc.
- The mechanism of action of the probiont and its effect on the general microflora.

Ideally, for probiotic candidates to be used in aquaculture they should:

- be members of the host's normal bacterial microflora.
- be able to colonise the host under the variable conditions present in the rearing system.

- have a beneficial effect on the host.
- be culturable in artificial medium on a large scale.
- maintain their viability and activity throughout production and storage.

A range of microorganisms has been suggested or evaluated for use in aquaculture; these include lactic acid bacteria (LAB) (Gatesoupe, 1994; Gildberg *et al.*, 1995; 1997; Jöbourn *et al.*, 1997; Robertson *et al.*, 2000), *Bacillus* species (Moriarty, 1998), *Pseudomonas* species (Gram *et al.*, 1999), and *Vibrio* species (Austin *et al.*, 1995; Ringø and Vadstein, 1998). However, there are some concerns over the use of organisms from taxa which have previously been associated with disease.

In aquatic organisms, the microflora is largely transient, unlike that of man and animals, which remains relatively constant, although the knowledge of bacterial diversity in the gastrointestinal tract of humans is still limited (Hold *et al.*, 2002). The temperature, salinity, oxygen concentration, intake of water through drinking and osmoregulation (Reitan *et al.*, 1998), and quantity and quality of feed are all determining factors that can affect the microflora of the fish.

Probiotics can be applied to the larval rearing systems by: (1) introducing specific bacteria into the digestive system via the live feed or inert diet (Munro *et al.*, 1995; 1999; Makridis *et al.*, 2000a); (2) adding beneficial bacteria to the rearing water (Ringø and Vadstein, 1998; Makridis *et al.*, 2000a); (3) adding naturally occurring compounds to the inert diet, which may selectively stimulate a beneficial microflora. As mentioned previously, the primary intestinal microflora establishes at the yolk-sac stage and is transient, and this is followed by bacterial successions until the adult microflora is established (Hansen and Olafsen, 1999; Ringø and Birkbeck, 1999).

The introduction of bacteria with the live food requires the bacterial strains to be bioencapsulated with the food. Optimisation of this bioencapsulation is necessary to achieve the correct level, so as not to affect the larval consumption rates. Turbot larvae have shown a decreased feeding rate when the rotifers fed had a high bacterial load (Perez-Benavente and Gatesoupe, 1988; Nicolas *et al.*, 1989; Gatesoupe, 1990). For successful colonisation it is important to determine the rate of loss of the bacteria

and whether there is any change in the microflora composition of the rotifers, as they may digest the loaded bacteria (Makridis *et al.*, 2000a). Therefore, through manipulation of the microflora in the live food, the rate and the type of colonisation can be influenced. Overall, it is important to control the level of colonisation of the turbot larvae as Munro *et al.* (1993) found there was a negative correlation between the rate of colonisation and the survival of turbot larvae.

The introduction of bacterial species to the rearing water at a very early stage can favour the growth of a protective flora, and thus further influence the development of the microflora (Strøm and Ringø, 1993; Munro *et al.*, 1995; Ringø and Vadstein, 1998; Huys *et al.*, 2001). However, the introduction of bacterial species to promote colonisation of the gut is a complex process. A range of parameters can affect the successful colonisation with a selected bacterial species including the presence of other bacterial species, whether they will colonise the gut, competition for attachment sites, and the developmental stage of the larvae (Ringø and Vadstein, 1998, Gomez-Gil *et al.*, 2000).

1.9.4.1. Potential Probiotics

Many groups have begun the search for effective probiotics to enhance the larval survival in finfish culture. As a result of the fish gut microflora playing a role in fish health, there is currently some interest in the manipulation of the gut flora towards a potentially more beneficial microbial community. There are no successful applications in large-scale rearing that have been published, although many groups have reported the identification of 'potential probionts'. With the considerable interest in the development of probiotics for use in aquaculture, methods applied to terrestrial animals have been tested, especially with regard to the use of lactic acid bacteria (LAB). Several reports exist of the use of LAB in larval rearing systems (Strøm and Ringø, 1993; Gatesoupe, 1994; Joborn *et al.*, 1997; Ringø *et al.*, 1999). However, as discussed in the microflora section, lactic acid bacteria are very minor components of the intestinal microflora of marine fish (Ringø and Gatesoupe, 1998).

At first-feeding, the microflora is dominated by the group *Vibrio/Aeromonas* (Bergh *et al.*, 1994). At this stage it is possible to induce an artificial dominance of

Lactobacillus/Carnobacterium by adding a strain isolated from rotifers, into the enrichment stage of the live feed (Gatesoupe, 1994). Gatesoupe (1994) used *Lactobacillus plantarum* isolated from rotifers as a probiotic for turbot larvae. The addition of this organism to rotifers increased their population density, reduced their bacterial loads and increased their dietary value. After challenge with *Vibrio* sp. the overall larval survival increased with no effect on the *Vibrio* levels in the tanks. Similar experiments with pollack, *Pollachius pollachius* showed that *Saccharomyces cerevisiae* and *Pediococcus acidilactici*, significantly enhanced growth but did not improve survival (Gatesoupe, 2002). Further examples of LAB that inhibit pathogens are a *L. lactis* AR1 strain bioencapsulated onto rotifers inhibited *V. anguillarum* (Harzevili *et al.*, 1998) and similarly a *L. plantarum* strain inhibited *A. salmonicida* (Gatesoupe, 1994).

In addition, a *Carnobacterium* sp. isolated from the intestine of Atlantic salmon produced *in vitro* antagonism against the fish pathogens *A. hydrophila*, *A. salmonicida* and *V. anguillarum* (Jöborn *et al.*, 1997). The strain adhered non-specifically to rainbow trout intestinal mucus and multiplied readily in the intestine of rainbow trout. Robertson *et al.* (2000) also carried out *in vitro* studies with a *Carnobacterium* sp. from Atlantic salmon intestine. Antagonistic activity was produced against *A. salmonicida*, *Photobacterium damsela*, *V. anguillarum* and *V. ordalii* but not against *V. alginolyticus*, *V. harveyi* or *Y. ruckeri*. Ringø *et al.* (2000) also found that a *C. piscicola* strain inhibited the growth of *A. salmonicida* *in vitro*.

However, *C. divergens* isolated from Atlantic salmon, was found to colonise the gut of larval turbot but no improvement in survival was shown (Ringø, 1999). Gildberg *et al.* (1995) also isolated lactic acid bacteria from salmon intestine and found no improvement in the resistance of Atlantic salmon (*Salmo salar*) fry when challenged with the pathogen *A. salmonicida*. The highest mortalities were in the fish given the lactic acid bacteria in their diet. In support of this, there have been reports of some lactic acid bacterial strains being pathogenic to fish. For example, *C. piscicola* was described as an opportunistic pathogen with low virulence likely to cause disease in stressed fish (Baya *et al.*, 1991).

In addition to LAB, other bacteria have been used. For example, Huys *et al.* (2001) exposed turbot larvae to a selection of bacteria. The larvae exposed to an isolate from sea bream larvae, *V. mediterranei* showed greater larval survival up to day 5 than the control larvae. In addition, a *Roseobacter gallaeciensis* strain isolated from a turbot larval rearing system was found to inhibit *V. anguillarum* and *V. splendidus in vitro* (Hjelm *et al.*, 2004). A *Roseobacter gallaeciensis* strain was also isolated from a scallop rearing unit with antagonistic activity towards a range of *Vibrio* species (Ruiz-Ponte *et al.*, 1998; 1999). Another example was reported by Gram *et al.* (1999) of a *Pseudomonas fluorescens* strain (AH2) that reduced the mortality of rainbow trout infected with *V. anguillarum*. Smith and Davey (1993) also found that Atlantic salmon psmolts treated with a fluorescent pseudomonad subsequently had reduced disease. However, in contrast the AH2 strain did not improve survival of Atlantic salmon cohabitants infected with furunculosis (Gram *et al.*, 2001).

1.9.4.2. Modes of Action

Suitable probiotic candidates should be able to survive, either within or outside of the host, be harmless to the organism being cultured and ideally be able to inhibit the growth of potential pathogens. There are various ways potential probionts can achieve this:

- by releasing antimicrobial substances
- by competition with the pathogen for potential receptor sites in the gut
- by stimulation of the immune system

However, the subject of probiotics remains controversial, primarily because the mechanisms by which probiotic organisms exert their action have not been identified and it is very unlikely that the beneficial effects of probiotics are the result of only one of these mechanisms.

1.9.4.2.1 Selection

The selection of potential probionts on a laboratory scale *in vitro* is important. However, rearing experiments *in vivo* are essential to demonstrate that the potential strains are not pathogenic (Hjelm *et al.*, 2004). Mechanisms of inhibition are largely dependent on the bacterial environmental conditions, therefore, *in vitro* and *in vivo*

assays may produce differing results. This was the case for the probiont *P. fluorescens* AH2 which had a strong *in vitro* antagonism but this was not replicated *in vivo* (Gram *et al.*, 2001). As Berg (1998) noted the constant feature of probiotic research is the huge variability in experimental results, especially in the *in vivo* studies.

1.9.4.2.2 Antagonistic bacteria

Antagonism is common in marine species (Gatesoupe, 1999). The main antagonists are normally members of the *Pseudomonas*-*Alteromonas* and/or *Vibrio* groups. This antagonistic effect may be mediated by antibiotics, inhibitory substances like organic acids, hydrogen peroxide, siderophores, lysozymes and proteases. Marine *Pseudoalteromonas* isolates produce inhibitory extracellular agents that have antibacterial and bacteriolytic activity (Holmström and Kjelleberg, 1999). The ECP of a *L. lactis* strain inhibited the proliferation of *V. alginolyticus* in *Artemia* and inhibited *V. alginolyticus* Hq221 isolated from diseased turbot (Villamil *et al.*, 2003), providing evidence that antagonistic factors can be released extracellularly.

Information concerning the growth-inhibiting effects of marine bacteria is very limited. Lemos *et al.* (1985) identified isolates from intertidal seaweed that had the ability to inhibit the growth of other bacteria. It was found that 38 out of 200 epiphytic isolates sampled from the seaweed had the ability to inhibit the growth of other bacteria. All the isolates were identified as belonging to the *Pseudomonas*/*Alteromonas* group. They were capable of inhibiting the growth of many fish pathogens, including *V. anguillarum* and *A. salmonicida* (Dopazo *et al.*, 1988). Therefore, this ability to produce compounds that inhibit bacterial growth may be widespread amongst marine bacteria (Bergh, 1995).

Indigenous bacteria isolated from the intestine and the skin-mucus of adult marine flat fish, turbot and dab (*Limanda limanda*) had the capacity to suppress the growth of *V. anguillarum* (Westerdahl *et al.*, 1991; Olsson *et al.*, 1992). Therefore, native bacteria found in fish intestines can be antagonistic against pathogenic vibrios and are very promising probiotic candidates since they are true colonisers of the gut. In similar work Ringø *et al.* (1996) isolated *V. pelagius* from healthy turbot and added it to the

rearing water of larval turbot. The addition of this strain was suggested to improve the survival of turbot larvae (Ringø and Vadstein, 1998), however, the survival data seems low. Bergh (1995) has shown that bacteria associated with first-feeding halibut larvae were capable of inhibiting the growth of larval pathogens. The frequency of growth-inhibiting isolates found was comparable to that of Westerdahl *et al.* (1991). Inhibitory bacteria belonging to the *Vibrio* spp. group were also isolated from the larval stages of the shellfish *Argopecten purpuratus* (Chilean scallop) (Riquelme *et al.*, 1997). Therefore, the use of bacterial strains with origins in fish, and particularly at the larval stage of rearing, may be the ideal way to isolate potential probiotic organisms.

1.9.4.2.3 Persistence

A successful antagonist may need to be present at significantly higher levels compared to the pathogen (Olsson *et al.*, 1998; Gram *et al.*, 1999). Inhibition of *V. anguillarum* in the faeces of turbot was only achieved when the number of viable cells of a probiotic *Carnobacterium* sp. was a hundred-fold greater than that of the pathogen (Jöburn *et al.*, 1997; Olsson *et al.*, 1998). Therefore, a potential probiont must ideally colonise the gut in order to replicate, or high levels of transient bacteria must be supplied on a regular basis for the probiotic activity to be exerted and maintained (Gram *et al.*, 1999).

Persistence in the gut would be advantageous, and Jöborn *et al.* (1997) found that a lactic acid *Carnobacterium* sp. that produced growth inhibitors against *V. anguillarum* and *A. salmonicida* *in vitro*, survived for several days in the intestine of larvae and juveniles. However this LAB strain was rapidly lost from the fish when the application stopped (Jöburn *et al.*, 1997). *Vibrio* sp. and *Aeromonas* sp. were also found to persist for days to weeks in turbot larvae (Munro *et al.*, 1995; Ringø and Vadstein, 1998; Gatesoupe, 1997). Nevertheless, Gatesoupe (1997) found that a *Vibrio* sp. loaded onto rotifers improved the survival of turbot larvae after 48 hours but unfortunately the effects did not persist.

1.9.4.2.4 Competition

Gatesoupe (1990) proposed that competition occurred between the bacterial strains of *V. alginolyticus* and *Aeromonas* species (later identified as *V. splendidus*, Gatesoupe, 1994) in turbot larvae. The *V. alginolyticus* strain dominated healthy larval tanks and the latter only proliferated in tanks on the verge of mortality. Competition for iron was suggested as the mechanism of inhibition for *P. fluorescens* against *A. salmonicida* (Smith and Davey, 1993) and for a *Vibrio* species that inhibited *V. splendidus* (Gatesoupe, 1997).

The black tiger shrimp (*Penaeus monodon*) treated with the probiont *Bacillus* S11 produced better survival when challenged with the pathogenic bacterium, *V. harveyi* (Rengpipat *et al.*, 2000). The mechanism of inhibition was thought to be as a result of activation of cellular and humoral immune defences as well as the possibility of competitive exclusion of the pathogen in the shrimp gut. The use of this probiont was most effective when it was applied at an early life stage and continued throughout culture.

Many different mechanisms involved in the probiotic process of competition and exclusion would be difficult for pathogens to overcome, thereby providing a successful microbial control mechanism. The use of probiotics should be considered a risk insurance that may not exert any beneficial effects when culture is performing well, but would be helpful if there were pathogens present or during disease outbreaks (Verschuere *et al.*, 2000b). However, there still remains a scarcity of data about the effective implementation of probiotics in practice and there is a great lack of knowledge about their exact mode of action. Therefore, questions have been posed regarding the success of probiotics in all systems (Berg, 1998).

1.9.5. Rearing water

1.9.5.1. Mature water

Marine environments are relatively rich in bacteria where nutrient availability, pH and temperature influence the bacterial flora. Bacteria take advantage of ecological changes introduced when seawater is used in aquaculture. Therefore, seawater can function as a growth medium and as a means of transport for microorganisms (Hansen

and Olafsen, 1999). Many bacteria have survival strategies that allow them to survive for months or even years in water and sediments (Hansen and Olafsen, 1999).

A highly artificial environment is created when fish are intensively produced in aquaculture, and this promotes bacterial growth. Water entering the culture system can undergo treatments such as filtration via membranes, UV sterilisation, chemical disinfection or antibiotic treatments (Salvesen *et al.*, 1999). These treatments can however, perturb and destabilise the bacterial community of rearing water, although, the treatment of inlet rearing water is regarded as a necessary measure to limit the microbial populations in the aquacultural systems (Salvesen *et al.*, 1999). After such treatments the rearing water is almost free of bacteria. However, with the combination of high larval densities, debris from dead larvae and high loads of organic material and bacteria from the addition of intensively produced live food, the proliferation of opportunistic bacteria with high growth rates is favoured which can lead to bacterial infections (Skjermo and Vadstein, 1999). Opportunistic fish pathogenic bacteria are common in seawater and can take advantage of the ecological changes introduced when water is used in aquaculture (Skjermo and Vadstein, 1999). Such opportunistic bacteria (r-strategists) are a continual problem in larval rearing as they out-compete the non-opportunistic strains (K-strategists) to cause disease. The r-strategists are capable of rapid growth favoured by nutrient rich conditions, and can form unstable populations with dominating 'pioneer' communities, whereas the K-strategists are slow growing organisms, favoured by the nutrient-limited conditions and can form stable mature communities that potentially exclude opportunists (Skjermo *et al.*, 1997).

The use of microbially matured seawater was suggested as an alternative method to the water treatments to control bacterial proliferation in the culture of marine larvae. The use of such mature water would lower the proliferation of opportunistic bacteria within the gut, thus leading to improved growth of the larvae (Vadstein *et al.*, 1993; Skjermo *et al.*, 1997; Salvesen *et al.*, 1999). By using a biomaturation filter the microflora of the water were adjusted to lower the levels of the r-strategists by reducing the level of nutrients to favour the growth of the K-strategists. In trials with a mature water system positive results on the growth rate and survival of Atlantic

halibut and turbot yolk-sac and first feeding larvae were achieved (Skjermo *et al.*, 1997; Skjermo and Vadstein, 1999; Salvesen *et al.*, 1999).

The use of microbially-mature water delays the rate of mucosal colonisation during first feeding and a slow rate of colonisation has been shown to be beneficial to turbot during gut development (Munro *et al.*, 1994). However, a problem observed by Salvensen *et al.* (1999) was that during hatching and first feeding, several factors like the addition of live food tended to counteract the stabilising effect of the water maturation and created environmental conditions that stimulate the growth of opportunists.

1.9.5.2. Green water

Another method to reduce microbes in the rearing system is to use microalgae. The microalgae are added to rearing system tanks to create 'green water' as well as being used as a live food enrichment for rotifers, which influences the gut microflora of the larval fish (Nicolas *et al.*, 1989; Skjermo and Vadstein, 1993; Bergh *et al.*, 1994; Reitan *et al.*, 1997; Olsen *et al.*, 2000). Salvesen *et al.* (1999) found that in addition to the use of matured seawater the application of microalgae to the rearing tanks greatly improved turbot growth.

In a study by Naas *et al.* (1992) the results did not indicate that the addition of microalgae had any direct or indirect nutritional effects. It was postulated that the addition of microalgae may have affected the microflora of the larvae as a result of better food uptake. It was proposed that the presence of suspended microalgae particles enhanced the visual contrast and therefore increased the visibility of the prey for the halibut larvae. The *Artemia* containing the algal cells appeared pigmented and therefore larvae may have been able to visualise them better. Enhanced feeding as a result of better visualisation of the prey was also shown in a study by Dendrinos *et al.* (1984) where stained *Artemia* improved the feeding efficiency of Dover sole larvae.

The beneficial effects of microalgae may arise from their exudates (Skjermo and Vadstein, 1993). Exudates produced by the microalgae *Tetraselmis suecica* have been found to have an inhibitory effect against the bacteria in the tank water and on the

walls of the tanks, accompanied by an increase in the number of bacteria in the digestive tract (Austin and Day, 1990; Austin *et al.*, 1992). The use of microalgae in the tanks of first-feeding summer flounder and in their live food produced a microflora that was dominated with non-vibrio heterotrophs (Eddy and Jones, 2002). This is in contrast to the first-feeding turbot fed with algal enriched rotifers which had a microflora dominated with vibrios (Munro *et al.*, 1994).

1.10. Summary of microbial control

To modify the gut microflora it is highly unlikely that a single bacterial species will prove effective as a probiotic. A combined approach of reducing the bacteria in the live food, colonising the live food with probiotics and stabilising the rearing water would all be required for rearing success. A consistent, beneficial gut flora in flatfish larval rearing has yet to be achieved.

1.11. Conclusion

Bacteria will remain to be a problem in any marine larval rearing system until there is strict control over the microbiological environment. This will not be achieved until more is known about the beneficial gut microflora. In addition to this the opportunistic bacteria associated with larval crashes need to be further identified and characterised in order to understand how the two types interact. With this knowledge and results from previous studies the work on microbial manipulation should successfully proceed to reduce the larval mortalities and increase aquaculture production overall.

1.12. Object of Research

Control of bacterial populations is of utmost importance within aquaculture systems as the proliferation of opportunists can cause high mortalities and subsequent 'crashes' of larvae. Although specific *known* pathogens have not been found to be associated with larval mortalities, there has been an association of haemolytic bacteria with significant losses of larval fish.

A selection of bacterial strains had been isolated from turbot larval 'crashes' at marine farm hatcheries by Thomson (2001) and haemolytic bacteria isolated from the live food, *Artemia* and turbot larvae were characterised. The majority of the haemolytic bacteria from the live food (*Artemia*) were identified as *Vibrio alginolyticus*, however the predominant haemolytic bacteria in the gut of the feeding larvae were *Vibrio splendidus* biovars 1 and 2. All strains pathogenic for turbot larvae belonged to the *V. splendidus* biovar 1 group.

The object of this research was to identify the virulence mechanisms associated with the pathogenic *V. splendidus* strains. In particular, the haemolysin of *V. splendidus* was studied to determine if it was a significant virulence factor that could cause damage in larval turbot.

Chapter 2. Characterisation of pathogenic bacteria

2.1. Introduction

Vibrio splendidus has been identified as a turbot larval pathogen in earlier studies (Gatesoupe *et al.*, 1999), and in this laboratory Thomson (2001) identified high numbers of virulent strains of *V. splendidus* biovar 1 from larval samples associated with larval crashes in turbot hatcheries in North-west Spain. *Vibrio splendidus* was found to be the dominant haemolytic bacterium associated with larvae feeding on *Artemia* and certain strains were identified as being pathogenic towards turbot larvae. The main aim of this thesis was to identify the virulence mechanisms of *V. splendidus*. Therefore, it was important to confirm the identity of the strain of *V. splendidus* biovar 1, using standard identification tests. Type strains of *V. splendidus* biovar 1 (NCIMB 1) and biovar 2 (NCIMB 2251) were used as reference strains.

2.2. Materials and Methods

2.2.1. Supply of bacteria

Vibrio splendidus DMC-1 was obtained from the culture collection of this laboratory and was originally isolated from moribund, larval turbot (Thomson, 2001). *Vibrio splendidus* NCIMB 1 and NCIMB 2251 were received from NCIMB, Aberdeen.

2.2.2. Culture and storage of bacteria

Bacteria were cultured on MA for 24 h or MB for 16-24 h at 20 °C. Strains were kept on MA plates at 4 °C for 2 weeks before being subcultured. For long-term storage the Protect system (Technical Service Consultants) was used at -80 °C with 100 µl of 20 % NaCl added to each storage vial to increase salinity to 3 % (w/v).

2.2.3. Gram stain and motility

Single colonies were selected for Gram stain (Madigan *et al.*, 2003). To determine motility, a drop of a 24-h culture grown in MB was placed on a microscope slide and a coverslip added. Using a phase contrast light microscope (Vickers Instruments) at x 1000 magnification motility was assessed visually.

2.2.4. Oxidase test

Filter paper strips were soaked in a 1 % solution of N, N, N, 'N'-tetramethyl-p-phenylenediamine dihydrochloride and allow to dry. The strip was inoculated with bacterial growth from 24 h MA plate using a sealed Pasteur pipette (nichrome wires should not be used as there is a possible risk of false positive reactions). Positive (*V. anguillarum*) and negative (*Escherichia coli*) controls were used and a deep purple colouration indicated a positive reaction.

2.2.5. TCBS agar

TCBS (Thiosulphate Citrate Bile-Salts) agar (Oxoid) was prepared according to the manufacturer's instructions. Single colonies from 24 h MA plates were streaked onto TCBS agar plates and incubated at 20 °C overnight (24 h). It was noted whether the bacteria utilised sucrose, indicated by an indicator dye colour change from green to yellow in the area surrounding the colony.

2.2.6. Catalase test

Bacterial growth from 24 h MA plates was spotted onto filter-paper strips and 1 drop of 30 % hydrogen peroxide (H_2O_2) added. A positive reaction was indicated by the rapid formation of bubbles after addition of the H_2O_2 .

2.2.7. Oxidation/Fermentation test

Hugh and Leifson (1953) medium with added 1.5 % NaCl was prepared in 5 ml aliquots and sterilised by autoclaving at 121 °C for 15 min. Filter-sterilised 10 % D-glucose solution was added to all tubes. To compare anaerobic and aerobic conditions, tubes were set up in duplicate and a liquid paraffin overlay added to one set after inoculation. The tubes were inoculated with bacteria from a single colony and incubated at 20 °C for 48-h. If both tubes changed colour from green to yellow then the organism was considered fermentative, but if only the aerobic tube changed colour then the organism was considered oxidative.

2.2.8. Haemolysis of sheep red blood cells

Bacterial strains were streaked onto 5 % sheep blood MA plates and were incubated at 20 °C overnight. Haemolytic activity was noted by the presence of clear zones around bacterial colonies. This was then measured semi-quantitatively using a

haemolysin plate assay. Blood cells were washed twice in sterile PBS and resuspended to 1 % v/v in PBS. Doubling dilutions of filter-sterilised bacterial supernatants from 16h MB cultures, were carried out in 100 μ l volumes in duplicate in a 96-well microtitre plate. Doubling dilutions from neat to 1/256 were set up in PBS and 100 μ l of blood suspension was added to each well and the plate incubated at 20 °C for 1 h to titrate the haemolytic activity. The end point of the titration was the highest dilution to yield approximately 50 % lysis of red blood cells. One haemolytic unit (HU) was defined as the amount of haemolysin which caused 50 % haemolysis in 200 μ l of a 0.5 % suspension of sheep erythrocytes. PBS was used as a negative control and saponin suspension as a positive control for haemolysis.

2.2.9. Lipase production on egg-yolk agar

The yolk was separated from the white of an egg and homogenised in 20 ml saline using a homogeniser. After centrifugation at 5,000 x g for 5 min the supernatant was used at 5 % v/v final concentration in TSA + 1.5 % NaCl. Bacteria were streak inoculated onto the plate and incubated at 20 °C for 48-h. Wells were also bored in the agar plate using a sterile cork borer and 20 μ l filter-sterilised bacterial supernatant from 16 h TSB + 1.5 % NaCl culture was added to each well. Plates were incubated for 4 days at 20 °C and a positive result was indicated by a zone of clearing in the agar.

2.2.10. Protease assay

2.2.10.1. Azocasein and azoalbumin

A 1 ml volume of 16 h MB bacterial culture was centrifuged and the supernatant retained. Trypsin (Bovine Pancreas, Type XIII, Sigma) standards (100 μ l), from 50 to 1500 μ g ml⁻¹, were set up using PBS as diluent and 100 μ l bacterial supernatant was added to eppendorf tubes at dilutions of neat and 1/10. A 100 μ l volume of 1 % azocasein or azoalbumin were added to each tube and mixtures were incubated at 37 °C for 30 min before 0.8 ml TCA was added to stop the reaction. Samples were centrifuged for 2 min in a bench centrifuge and 0.5 ml of supernatant was transferred to these tubes containing 0.5 ml of 0.5 M NaOH. Absorbance was measured at 440 nm with PBS as blank. Protease concentration was determined as the equivalent in μ g

ml⁻¹ trypsin (12,000 units/mg protein) from a plot of trypsin concentration against A440 nm and indicated as being positive or negative.

2.2.11. DNase production

DNase test agar (Becton Dickinson) with added 1 % NaCl was used according to the manufacturer's instructions, and autoclaved at 121 °C for 15 min. Filter-sterilised toluidine blue, 2 ml 0.01 % aqueous solution, was added to the molten agar. Plates were inoculated with 100 µl of 10⁻⁶ and 10⁻⁸ dilutions of MB 16h bacterial cultures and plates were incubated at 20 °C for 1 week. Positive reactions showed pink halos around the bacterial colonies.

2.2.12. Antibiotic sensitivity

Each isolate was screened against a range of antibiotics using the Mast Ring S system (Mast Diagnostics Ltd). Sensitive isolates showed a zone of clearing ≥ 2 mm around the antibiotic disc. An overnight (16 h) bacterial MB culture (100 µl) was spread onto MA plates and allowed to dry before applying the Mastring disc using sterile forceps. Plates were incubated at 20 °C overnight. A zone of inhibition of growth around an antibiotic disc indicated that the strain was sensitive to the antibiotic.

Mastring S antibiotics

The following antibiotics, and quantities/disc were used: (chloramphenicol, 50 µg; chloramphenicol, 25 µg; streptomycin, 25 µg; streptomycin, 10 µg; ampicillin, 25 µg; kanamycin, 30 µg; tetracycline, 100 µg; tetracycline, 25 µg; nalidixic acid, 30 µg; erythromycin, 5 µg; methicillin, 10 µg; penicillin G, 1 Unit; fusidic acid, 10 µg; novobiocin, 5 µg; colistin sulphate, 25 µg; nitrofurantoin, 50 µg).

2.2.13. API-ZYM

Bacteria were grown overnight for 16 h in a 10 ml MB culture to obtain an OD₆₀₀ of ~0.5-0.7 and cultures washed twice in SSW and resuspended in SSW to obtain a cell concentration of 2 x 10⁹ cells ml⁻¹. An incubation tray was set up with water in the base to provide a humid atmosphere and 65 µl of the bacterial suspensions added to wells of the API-ZYM strip (bioMérieux) prior to incubation at 20 °C for 4 h with the incubation tray lid on. After incubation, one drop of ZYM A and ZYM B reagents

was added and colour developed by exposing the strip to daylight. Negative reactions remained colourless. Using a colour shade chart provided by the manufacturer a value ranging from 0-5 was assigned corresponding to the colour that developed: 0 corresponded to a negative reaction, 5 to a reaction of maximum intensity and values 1-4 were intermediate reactions with values 3-5 being considered as positive reactions (see below).

0 = negative control

1 = Liberation of 5 nmoles product

2 = 10 nmoles

3 = 20 nmoles

4 = 30 nmoles

5 = ≥ 40 nmoles

2.2.14. Biolog

Single carbon source utilisation using Biolog plates (Oxoid) was measured. Colonies from a MA plate were inoculated into 5 ml MB and were grown overnight (16 h) at 20 °C. Cells were harvested and washed in SSW to remove MB components, when they had reached an OD₆₀₀ of 0.8-1.2. The pellet was resuspended in an equal volume of Biolog inoculation medium (2.5 % NaCl, 0.05 % KCl, 0.8 % MgCl₂, 0.15 % carageenan) and the OD₆₀₀ was measured. An OD₆₀₀ of 0.1 was required for inoculation into the 96-well Biolog plate, and cultures were diluted to the required OD using Biolog inoculation medium; 150 µl of bacterial suspension was inoculated into the Biolog plates which were then incubated at 20 °C for 48 h. Results were recorded using the values of 0, 2, 5, and 10 dependent on the intensity of purple coloration.

2.3. Results

2.3.1. Basic biochemical tests

Vibrio splendidus DMC-1 and the 2 type strains were motile, fermentative, oxidase and catalase positive, Gram-negative rods, which grew on TCBS (Table 2.1).

Enzyme activity was recorded as positive or negative.

TEST	DMC-1	NCIMB-1	NCIMB-2251
Gram Stain	0	0	0
Motility	1	1	1
Oxidase	1	1	1
Catalase	1	1	1
Oxidative/Fermentative	F ¹	F	F
TCBS	1	1	1
Sucrose	0	0	0
Haemolysin -cells	1	1	1
-supernatant	1	1	1
Phospholipase -cells	1	1	0
-supernatant	1	1	0
Protease (Azocasein) -cells	1	1	0
-supernatant	1	1	0
Protease (Azoalbumin) -cells	1	1	0
-supernatant	1	1	0
DNase	1	1	1

Table 2.1. Basic biochemical test results of *V. splendidus* strains. 1 = Positive, 0 = Negative. ¹Fermentative.

2.3.2. Antibiotic sensitivity

Overall, the antibiotic sensitivity profiles for the *V. splendidus* strains showed *V. splendidus* DMC-1 had 94 % identity with biovar 1 and 81 % identity with biovar 2 (Table 2.2).

Antibiotic resistance	DMC-1	NCIMB-1 biovar 1	NCIMB-2251 biovar 2	Matches ¹
Chloramphenicol 50 µg	0	0	0	Biovar 1&2
Chloramphenicol 25 µg	0	0	0	Biovar 1&2
Streptomycin 25 µg	1	1	0	Biovar 1
Streptomycin 10 µg	1	1	0	Biovar 1
Ampicillin 25 µg	1	1	1	Biovar 1&2
Kanamycin 30 µg	0	0	0	Biovar 1&2
Tetracycline 100 µg	1	1	1	Biovar 1&2
Tetracycline 25 µg	1	1	1	Biovar 1&2
Nalidixic acid 30 µg	0	1	0	Biovar 2
Erythromycin 5 µg	1	1	0	Biovar 1
Methicillin 10 µg	1	1	1	Biovar 1&2
Penicillin G 1 Unit	1	1	1	Biovar 1&2
Fusidic acid 10 µg	1	1	1	Biovar 1&2
Novobiocin 5 µg	0	0	0	Biovar 1&2
Colistin sulphate 25 µg	0	0	0	Biovar 1&2
Nitrofurantoin 50 µg	0	0	0	Biovar 1&2

Table 2.2. Antibiotic sensitivity profiles of *V. splendidus* strains. 1 = Resistant, 0 = Sensitive. ¹Comparison of *V. splendidus* DMC-1 with type strains.

2.3.3. API-ZYM

API-ZYM profiles of the *V. splendidus* strains showed 100 % identity between *V. splendidus* DMC-1 and biovar 1, and 79 % identity with biovar 2 (Table 2.3).

API-ZYM	DMC-1	NCIMB-1 biovar 1	NCIMB-2251 biovar 2	Matches with DMC-1 reaction ¹
Alkaline phosphatase	1	1	1	Biovar 1&2
Esterase (C4)	1	1	1	Biovar 1&2
Esterase Lipase (C8)	1	1	1	Biovar 1&2
Lipase (C14)	0	0	0	Biovar 1&2
Leucine arylamidase	1	1	1	Biovar 1&2
Valine arylamidase	0	0	0	Biovar 1&2
Cystine arylamidase	0	0	0	Biovar 1&2
Trypsin	1	1	1	Biovar 1&2
α -chymotrypsin	0	0	0	Biovar 1&2
Acid phosphatase	1	1	1	Biovar 1&2
Naphthol-AS-BI- phosphohydrolase	1	1	0	Biovar 1
α -galactosidase	0	0	0	Biovar 1&2
β -galactosidase	1	1	0	Biovar 1
β -glucuronidase	0	0	0	Biovar 1&2
α -glucosidase	1	1	0	Biovar 1
β -glucosidase	0	0	0	Biovar 1&2
N-acetyl- β -glucosaminidase	1	1	0	Biovar 1
α -mannosidase	0	0	0	Biovar 1&2
α -fucosidase	0	0	0	Biovar 1&2

Table 2.3. API-ZYM profiles of *V. splendidus* strains. 1 = Positive, 0 = Negative.

¹Comparison of *V. splendidus* DMC-1 with type strains.

2.3.4. Biolog

Biolog profiles for all three organisms were compared and *V. splendidus* DMC-1 showed 93 % and 87 % identity with *V. splendidus* biovars 1, and 2, respectively (Table 2.4).

BIOLOG Tests	DMC-1	NCIMB-1	NCIMB-2251	Matches with DMC-1 reaction ¹
α -cyclodextrin	0	0	0	Biovar 1&2
dextrin	10	10	10	Biovar 1&2
glycogen	10	10	10	Biovar 1&2
tween 40	10	10	10	Biovar 1&2
tween 80	10	5	10	Biovar 1&2
n-acetyl-d-galactosamine	0	0	0	Biovar 1&2
n-acetyl-d-glucosamine	10	10	10	Biovar 1&2
adonitol	0	0	0	Biovar 1&2
L-arabinose	0	0	0	Biovar 1&2
d-arabitol	0	0	0	Biovar 1&2
cellobiose	10	10	10	Biovar 1&2
L-erythritol	0	0	0	Biovar 1&2
d-fructose	10	10	10	Biovar 1&2
L-fucose	0	0	0	Biovar 1&2
D-galactose	10	10	0	Biovar 1
gentiobiose	10	5	0	Biovar 1
α -d-glucose	10	10	10	Biovar 1&2
m-inositol	0	0	0	Biovar 1&2
α -lactose	10	0	0	- ³
α -d-lactose-lactulose	0	0	0	Biovar 1&2
maltose	10	10	10	Biovar 1&2
d-mannitol	10	10	10	Biovar 1&2
d-mannose	10	10	0	Biovar 1
d-melibiose	5	0	0	-
β -methyl-d-glucoside	0	0	0	Biovar 1&2
psicose	2	0	2	Biovar 2
d-raffinose	0	0	0	Biovar 1&2
l-rhamnose	0	0	0	Biovar 1&2
d-sorbitol	2	0	0	-
sucrose	0	0	0	Biovar 1&2
d-trehalose	10	10	10	Biovar 1&2
turanose	0	0	0	Biovar 1&2
xylitol	0	0	0	Biovar 1&2
methyl pyruvate	10	2	10	Biovar 1&2
mono-methyl succinate	0	2	10	-
acetic acid	10	5	10	Biovar 1&2
cis-aconitic acid	2	5	10	Biovar 1&2
citric acid	10	5	10	Biovar 1&2
formic acid	0	2	0	Biovar 2
D-galactonic acid lactone	0	0	0	Biovar 1&2
D-galacturonic acid	0	0	0	Biovar 1&2
D-gluconic acid	10	10	0	Biovar 1
D-glucosamic acid	0	0	0	Biovar 1&2
D-glucuronic acid	10	10	0	Biovar 1
α -hydroxy butyric acid	0	0	0	Biovar 1&2
β -hydroxy butyric acid	0	0	0	Biovar 1&2
γ -hydroxy butyric acid	0	0	0	Biovar 1&2
p-hydroxy phenylacetic acid	0	0	0	Biovar 1&2
itaconic acid	0	0	0	Biovar 1&2
α -keto butyric acid	0	0	0	Biovar 1&2

α -keto glutaric acid	10	10	10	Biovar 1&2
α -keto valeric acid	0	0	0	Biovar 1&2
D, L-lactic acid	10	10	10	Biovar 1&2
malonic acid	0	0	0	Biovar 1&2
propionic acid	10	10	0	Biovar 1
quinic acid	0	0	0	Biovar 1&2
D-saccharic acid	0	0	0	Biovar 1&2
sebacic acid	0	0	0	Biovar 1&2
succinic acid	10	10	10	Biovar 1&2
bromo-succinic acid	5	5	10	Biovar 1&2
succinamic acid	0	0	0	Biovar 1&2
glucuronamide	0	0	0	Biovar 1&2
alaninamide	0	0	0	Biovar 1&2
D-alanine	10	10	10	Biovar 1&2
L-alanine	10	10	10	Biovar 1&2
L-alanyl glycine	10	10	10	Biovar 1&2
L-asparagine	10	10	10	Biovar 1&2
L-aspartic acid	10	10	10	Biovar 1&2
L-glutamic acid	10	10	10	Biovar 1&2
Glycyl-L-aspartic acid	10	10	10	Biovar 1&2
Glycyl-L-glutamic acid	10	10	10	Biovar 1&2
L-histidine	2	0	10	Biovar 2
hydroxy L-proline	0	0	0	Biovar 1&2
L-leucine	0	0	0	Biovar 1&2
L-ornithine	0	0	2	Biovar 1
L-phenyl alanine	0	0	0	Biovar 1&2
L-proline	10	10	10	Biovar 1&2
L-pyro glutamic acid	0	0	0	Biovar 1&2
D-serine	0	0	0	Biovar 1&2
L-serine	10	10	10	Biovar 1&2
L-threonine	10	10	10	Biovar 1&2
D, L-carnitine	0	0	0	Biovar 1&2
γ -amino butyric acid	0	0	0	Biovar 1&2
urocanic acid	0	0	0	Biovar 1&2
inosine	10	10	10	Biovar 1&2
uridine	10	10	10	Biovar 1&2
thymidine	10	10	10	Biovar 1&2
phenyl ethylamine	0	0	0	Biovar 1&2
putrescine	0	0	0	Biovar 1&2
2-amino ethanol	0	0	0	Biovar 1&2
2, 3 butanediol	0	0	0	Biovar 1&2
glycerol	10	10	10	Biovar 1&2
D, L- α -glycerol phosphate	10	10	10	Biovar 1&2
glucose-1-phosphate	10	10	0	Biovar 1
glucose-6-phosphate	10	10	10	Biovar 1&2

Table 2.4. Biolog profiles of *V. splendidus* strains. The reactions were scored as follows: 10, very strong; 5, strong; 2, weak; 0, no reaction. ¹Comparison of *V. splendidus* DMC-1 with type strains. ²No match with biovar 1 or 2.

2.4. Discussion

Vibrio splendidus is commonly considered to be an environmental organism of no pathogenic importance (Lavillapitogo *et al.*, 1990, Myhr *et al.*, 1991). However, several studies have reported that it is involved in infections in turbot (Myhr *et al.*, 1991; Pazos *et al.*, 1993; Angulo *et al.*, 1994b; Gatesoupe, 1999; Thomson, 2001). The organism isolated by Thomson (2001), strain DMC-1, was confirmed as *V. splendidus* biovar 1. This was in agreement with the 16S partial sequence of the rRNA gene determined for this strain (Thomson, 2001).

According to Bergey's manual of Determinative Bacteriology (Holt *et al.*, 1994), 11-89 % of *V. splendidus* biovar 1 strains are positive for sucrose utilisation but this trait is negative for biovar 2. Therefore, it would be expected that the biovar 1 type strain would be sucrose positive; however, in Biolog GN plates and on TCBS plates negative results were obtained. Strain DMC-1 that has identity with biovar 1 was likewise sucrose negative.

In the antibiotic profiles it was expected that DMC-1 would have different resistance profiles to the other two strains as it was isolated from a fish farm where antibiotics were used to control bacterial levels within the rearing system. However, the DMC-1 profile showed 94 % identity to biovar 1, suggesting that DMC-1 was a recent introduction to the larval rearing system, perhaps via the *Artemia* live feed.

In API-ZYM, DMC-1 showed a 100 % match with biovar 1. In a study by Gatesoupe *et al.* (1999) on *V. splendidus* isolates pathogenic for larval turbot it was found that valine aminopeptidase was produced only by the pathogenic group of isolates and was thought to be a virulence marker. Valine arylamidase was one of the enzymes assayed in the API-ZYM kit and all three *V. splendidus* strains were negative for this enzyme showing that there is no association of this enzyme with virulence in the pathogenic DMC-1 strain.

Most of the results obtained in the Biolog GN assays were in agreement with published results for the use of particular substrates for type strains of biovars 1 and 2 (Holt *et al.*, 1994), although not all the substrates tested for in Biolog are listed in

Bergey's Manual. Firstly the differences in Biolog reactions found between DMC-1 and biovars 1 and 2 included DMC-1 giving positive reactions for α -lactose, d-melibiose and d-sorbitol, and a negative reaction for mono-methyl succinate (Holt *et al.*, 1994).

The differences found between biovars 1 and 2 are those which discriminate between these biovars, according to Bergey's Manual. Differences found with Biolog for biovar 1 include L-histidine utilisation which gave a negative reaction, but is usually positive in 11-89 % of biovar 1 strains (Holt *et al.*, 1994). Other differences were noted for biovar 2 in the utilisation of L-ornithine and propionic acid which had Biolog scores of 2 and 0 respectively whereas Biovar 2 strains should have produced the opposite results. If the very weak reactions (scores of 2) had been classed as negative reactions, DMC-1 would have shown higher identity to biovar 1, giving matches with psicose, d-sorbitol, and mono-methyl succinate. At the same time, Biolog scores of 2 were obtained for biovar 1 strains in utilisation of pyruvate, (methyl-pyruvate) whereas these should be positive according to Bergey's Manual (Holt *et al.*, 1994).

In summary, it was concluded that the identification of strain DMC-1 as *V. splendidus* biovar 1 was correct and that the strain was closely related to the type strain.

Chapter 3. Pathogenicity of *V. splendidus* DMC-1 towards cells and turbot larvae.

3.1. Introduction

To confirm the pathogenicity of *V. splendidus* DMC-1 it was necessary to use *in vivo* trials but *in vitro* assays for potential virulence factors were also used. The bivalve haemocyte cytotoxicity assay is a simple *in vitro* assay that has been used to screen bacteria associated with larval mortalities to identify which are highly cytotoxic. (Lane and Birkbeck, 1999). In this study it was employed to assess the cytotoxicity of bacterial strains isolated from turbot larvae, including *V. splendidus*. Another *in vitro* assay used turbot macrophages based on the protocol for the haemocyte assay with modifications (Lambert and Nicolas, 1998). Turbot tissue culture cells TV1-S4 (Fernández-Puentes *et al.*, 1993) and TF-1 (provided by Keith Way, CEFAS, Weymouth, U.K.) were also used in cytotoxicity assays to provide a more relevant cell target for the isolates in question.

Once *in vitro* screening of cytotoxicity had been done, *in vivo* challenge trials were carried out using both yolk-sac and first feeding turbot larvae. Challenge trials against turbot larvae have been reported for bacterial strains by Shiftesvik and Bergh (1993), Munro *et al.* (1995), Bergh *et al.* (1997) and Ringø and Vadstein (1998). More importantly, *V. splendidus* strains have been shown to be pathogenic against turbot larvae by Gatesoupe *et al.* (1999) and Thomson (2001).

3.2. Materials and Methods

3.2.1. Haemocyte assay

The common mussel, *Mytilus edulis* were kept in aquarium tanks at 20 °C and were fed on algae, *Pavlova lutheri* at 20 °C. Haemolymph was collected from the adductor muscle using a 1 ml syringe and a 26 gauge needle, and haemocytes were counted using a Neubauer counting chamber. Haemolymph (30 µl) was added to the wells of a 96-well flat-bottomed microtitre plate and haemocytes were allowed to adhere to the surface of the plate for 1 h at 20 °C. The adherent cells were gently washed with SSW to remove non-adherent cells and washed bacteria suspended in filtered haemolymph were applied at a ratio of 50 bacteria per haemocyte in a final volume of 200 µl. Sterile-filtered bacterial supernatant was also tested in the assay (200 µl per well). Control haemocytes were overlaid with filter-sterilised haemolymph containing no

bacteria. In the assays, *Vibrio splendidus* DMC-1, LTS-3 and DTC-5 were used as the challenge strains with a highly cytotoxic strain, *V. anguillarum* 2981 and a non-toxic strain, *Alteromonas* 1-1-1. (NCIMB 2024) being used as the positive and negative control strains, respectively. The haemocytes/bacteria (or supernatant) mixtures were incubated at 20 °C for 3 h and cytotoxicity was recorded as the percentage of rounded haemocytes determined from the proportion of spread and round cells. Bacteria were tested in duplicate with counts taken from two fields of view for each well. Counts were normalised to take into account the proportion of rounded cells in the control wells, using the formula:

$$\text{Cell rounding} = (T-C)/(100-C) \times 100$$

Where T = % rounded cells in test sample and C = % cells rounded in control samples.

All bacterial strains used in the haemocyte assay were grown in baffled 250 ml Erlenmeyer flasks from a single colony inoculation into 50 ml of MB at 20 °C for 24 h.

3.2.2. Haemolysin and haemagglutination assays

Sheep erythrocytes were the main blood cells used throughout this work, however, rabbit, horse, turbot and salmon erythrocytes were also tested. Blood was washed twice in PBS, or PBS + 2 % NaCl for the latter two blood types, and erythrocytes resuspended to 1 % v/v in buffer. Filter-sterilised bacterial MB supernatant (100 µl), or washed bacterial cells (approx. 10^9 cells ml⁻¹, 16 h growth) (100 µl), were added to the wells of a 96-well multititre plate according to section 2.2.8. The 50 % end point was determined visually but for some experiments the end point was determined spectrophotometrically. For the latter method the plate was set up as above with agitation during the incubation step so that blood cells remained in suspension. After incubation light scattering was measured at 620 nm in a Rosys Anthos 2001 plate spectrophotometer and the 50 % end point determined. Both haemolysin and haemagglutination titres were measured.

3.2.3. Turbot erythrocyte cell assays

Turbot blood cells were obtained from freshly collected blood samples of adult turbot fish from Ardtaraig (Aquascot) and Ardtoe (Scafish Industry Authority). Blood was collected into an equal volume of 3.8% sodium citrate and was transported to the

laboratory on ice. Between 0.5 ml and 1 ml of citrated blood was centrifuged at 5,000 x g for 5 min and the pellet of erythrocytes suspended in PBS, and washed twice with 1 ml sterile PBS + 2 % NaCl. A 1 % v/v suspension of erythrocytes was prepared in PBS + 2 % NaCl. MB bacterial suspensions grown for 16 h at 20 °C were harvested at 10,000 x g for 10 min and supernatant was filter-sterilised and held on ice while the bacterial cells were washed twice in PBS + 2% NaCl and resuspended to 5×10^9 cells ml⁻¹. The washed bacterial suspensions and bacterial supernatant (100 µl) were added to a 96-well microtitre plates. The bacterial cells and the supernatant were diluted using doubling dilutions (1/2 to 1/64) in PBS + 2 % NaCl. The washed turbot erythrocyte suspension was added to each well (100 µl) and incubated at 20 °C for 1 h. The haemolysis and haemagglutination titres were measured.

3.2.4. Turbot macrophage assay

3.2.4.1. Cytotoxicity assay

Turbot head kidneys were isolated from adult turbot fish at Ardtaraig or Ardtoe and were kept on ice during transport to the laboratory. Using aseptic techniques the kidneys were cut into small sections using a scalpel and placed into glass homogenisers with 5 ml 1 x HBSS with heparin (10 U heparin ml⁻¹). The tissue was gently homogenised and the cell suspension allowed to settle to remove aggregates. The suspended cells were transferred to plastic universals and 34 % v/v Percoll was applied below the suspension with a sterile syringe and needle. This was followed by the application of a layer of 51 % v/v Percoll below the layer to generate a discontinuous gradient. The suspension was then centrifuged at 400 x g for 20 min at 4 °C. The band of cells at the 34%: 51% interface, enriched with macrophage, was collected and washed with 1x HBSS. The supernatant layer was carefully removed and the cell pellet resuspended in 20 ml 1x HBSS. A 20 µl sample of cells was stained with 0.2 % trypan blue to assess viability and to obtain a cell count. Cells were added to the wells of a 24-well tissue culture plate at a density of 8.7×10^5 cells per well. The plate was incubated at room temperature for 1 h to allow cells to adhere, after which the plate was washed vigorously with 1x HBSS before addition of 500 µl filter-sterilised bacterial supernatants at dilutions of neat, 1/10 and 1/100, using 1x HBSS as diluent. Plates were incubated at room temperature for 2-3 h before cells were fixed with methanol (0.5 ml per well) for 10 min. Methanol was removed and the same volume of Giemsa stain was added for 10 min. Plates were rinsed twice

with PBS, air-dried and cell numbers visually assessed using an inverted microscope. The number of assays was limited due to the availability of fresh turbot head kidneys.

3.2.5. Tissue culture assays

Turbot tissue culture cell lines were obtained from José Fernández-Puertes, University of Santiago, Spain (TV1-S4) and Keith Way, CEFAS, U.K. (TF-1). Both turbot cell lines were used initially to assess the toxicity of *V. splendidus* DMC-1 cells and supernatant. Tissue culture cells were treated with filter-sterilised supernatant from a 24 h culture of DMC-1. Doubling dilutions were set up in Eagles Minimal Essential Medium (MEM) at dilutions up to 1/128. Assay plates were examined using an inverted microscope and cells were assessed visually for cytopathic effects. A tetrazolium reduction assay using the tetrazolium salt, XTT (Sigma) was used to measure the cell activity after incubation with concentrated bacterial supernatant (dilutions of neat to 1/2560). These salts are reduced by dehydrogenases to coloured tetrazolium formazan products. After 24-h incubation with the concentrated bacterial supernatant the suspensions were removed from the tissue culture plate and XTT, dissolved in tissue culture medium was added to each well at a final concentration of 0.25 mg ml⁻¹. A menadione solution (1 mM in acetone) was added to the wells of the XTT assay plate at a final concentration of 1 µM. The plate was incubated at 18 °C for 4-h. The solutions were removed from the wells and clarified by centrifugation, before the concentration of the formazan product was measured at 492 nm in a spectrophotometer.

3.2.6. Turbot yolk-sac larval challenge trial carried out in Bergen.

3.2.6.1. Infection of turbot larvae

Turbot eggs were obtained from a commercial hatchery (Stolt Sea Farm, Kvinesdal, Norway) and transported to the challenge laboratory of the Institute of Marine Research (IMR, Bergen, Norway) 1.5 to 2 days prior to hatching. Transportation was by car and aeroplane, and transport time from hatchery to laboratory was approximately 7 h. The incubation temperature during transport was approximately 12 °C and the challenge procedure was modified from Bergh et al. (1992; 1997). Seawater was diluted with distilled water to 30 ‰ salinity and autoclaved; 2 ml volumes of this were distributed into the wells of 24-well polystyrene dishes (Nunc, Roskilde, Denmark). Upon arrival, the eggs were incubated in a temperature-

controlled room at 16 °C and were distributed individually into each well of the polystyrene dishes. Immediately after distributing eggs, 100 µl of bacterial suspensions at 1×10^6 and 1×10^8 cells ml⁻¹ for trial 1, and 1×10^5 and 1×10^7 cells ml⁻¹ for trial 2, were added to the wells. Seventy-two larvae (three multiwell dishes) were assigned to each concentration of bacteria. In addition, an unexposed control group was included. As the survival of each individual larva was independent of the survival of larvae in other wells, it was assumed that the data were binomially distributed. Differences between mortalities (on single days) between treatments were tested by a Chi-square contingency table test using the Minitab program. Survival or death of each individual larva was recorded for 6 days post-hatch. No exchange of water was carried out during the experiment.

3.2.6.2. Challenge bacteria

A starter culture of *V. splendidus* DMC-1 was grown for 24 h in 20 ml of Marine Broth (Difco 2216) and 2 % (400 µl) of the starter culture was inoculated into 20 ml of fresh MB and grown for 24 h at 20°C with moderate shaking at 100 rpm. Cell concentrations of bacterial suspensions were tentatively calculated from OD measurements of exponentially growing cultures and diluted in 30 ‰ autoclaved seawater. Cells were then washed twice in 30 ‰ autoclaved seawater prior to inoculation into the larval wells to final concentrations of 1×10^6 and 1×10^8 cfu ml⁻¹ for trial 1 and 1×10^5 and 1×10^7 cells ml⁻¹ for trial 2. Cell concentrations were verified by plate counting on MA, incubated at 20 °C.

3.2.7. First-feeding larval trial carried out at Bergen

3.2.7.1. Turbot larvae

Turbot larvae (1 d post hatch) were received from a commercial hatchery (Stolt Sea Farm, Kvinesdal, Norway) having been transported to the Institute of Marine Research by car and aeroplane, with a journey time of approximately 7 h. Full strength (32 ‰ salinity) seawater in 160 L tanks was aerated with double air-stones (1 cm x 4 cm) and prior to addition of larvae, an algal concentrate of *Nannochloropsis* sp. (Reed Mariculture, USA) was added to create a "green water" system.

Larvae were transferred into tanks on day 1 post-hatch to give approximately 1500 larvae per tank and temperature was maintained between 15.7 and 17.1 °C. Four

tanks were set up initially, but the population in tank 1 crashed on day 2. Therefore, only one control group was present in this trial.

3.2.7.2. Algae and rotifers

Nannochloropsis (Reed Mariculture, USA) was used from a frozen concentrated stock supplied from the Institute for Fish and Marine Biology, University of Bergen and 0.5 g was added to tanks on days 1-3 and 6-8. Rotifers, *Brachionus plicatilis* (13 L @ 600 ml⁻¹) were supplied by Julie Skadal, University of Bergen and rotifers were cultured and maintained in 80 L conical tanks using 42 L of 22 ‰ salinity at 24 °C. Rotifers were maintained by daily feeding with 1 gram fresh yeast per million rotifers and 0.1 g DC DHA Selco (INVE) per gram of yeast. Numbers were counted and recorded daily.

3.2.7.3. Bacteria

These were as before, (section 3.2.6.2).

3.2.7.4. Colonisation of rotifers

Rotifers were siphoned from the growth vessel, and rinsed with 22 ‰ salinity seawater and concentrated in 250ml beakers to the required number for daily feeding to the turbot larvae from day 2 post-hatch. Bacteria were added to give a final cell density of 1×10^7 cfu ml⁻¹ (actual concentrations found by plate counts were: DMC-1 = 5×10^7 and 7×10^7 cfu ml⁻¹; on the two challenge days) and were incubated with the rotifers for 1 h at 20 °C. Inoculated rotifers were fed to the larvae at a concentration of 2 rotifers ml⁻¹ on days 4 and 5 post-hatch and thereafter the density of rotifers was maintained at 4-6 rotifers ml⁻¹ by daily addition.

3.2.7.5. Sampling of larvae for immunohistochemistry

Larvae were sampled daily and fixed in phosphate buffered 3.7 % formaldehyde (pH 7.0.) The larvae were dehydrated through a graded ethanol series and embedded in paraffin. Sections were cut (3 µm thick) on a Reichert-Jung Biocut, incubated for 30 min at 56 °C, de-waxed in xylene, rehydrated through a graded ethanol series (100 %, 96 %, 70 %, 50 %), and finally into distilled water. Nonspecific antibody binding sites were blocked by covering the sections with a solution of 5 % bovine serum albumin (BSA, Sigma, UK) in Tris-buffered saline (TBS, pH 7.4) for 20 min. Slides

were incubated with the primary rabbit antiserum against *Vibrio splendidus* DMC-1 (Harland Seralab, UK) at a dilution of 1:900 in 2.5 % BSA TBS for 30 min. After washing for 5 min in TBS, the secondary antibody, biotinylated goat anti-rabbit immunoglobulin, diluted 1:300 in 2.5 % BSA TBS (Dakopatts) was added and incubated at room temperature for 30 min. After washing in TBS, streptavidin alkaline phosphatase complex was added, and incubated for 30 min. After washing, New Fuchsin Chromogen K698 (Dako) with 1 mM levamisole (Sigma) in TBS was added and allowed to develop for 5 min. After washing with water, sections were counterstained with Mayer's haematoxylin and were mounted in an aqueous mounting medium (Aquamount-BHD). All incubations were performed at room temperature (approximately 20 °C) in a humidity chamber. As controls, tissue sections from both the control larvae (not exposed to pathogen), and exposed larvae were incubated with immune and non-immune (normal rabbit) serum.

Sections were examined by light microscopy and photographs were taken using a camera attachment to the microscope.

3.2.8. First-feeding larval trial carried out in Glasgow

3.2.8.1. Turbot larvae

Turbot larvae were received from Stolt Sea Farms, Spain at 3 days post-hatch by air and were transported by car to the laboratory. The total transportation time was about 48 hours.

Larvae were held in a temperature controlled environment room (CER) at 16 °C. To ensure feeding commenced, the larvae were held together until day 5 post-hatch. Rotifers were given to larvae at 1-2 ml⁻¹ on these days and increased to 4-6 ml⁻¹ thereafter. Tanks were constantly illuminated and gently aerated for the duration of the trial (Figure 3.1). Algae (*Nannochloropsis*) were added to give 'green water' in the tanks, and this was maintained on a daily basis. One third of the tank water was exchanged every second day after day 5 post-hatch.



Figure 3.1. Larval trial in controlled environment room. Fifty turbot larvae were held in 3 L of seawater in triplicate for each group. Tanks were aerated and illuminated as shown.

On day 5 post-hatch, larvae were transferred into 5 L beakers containing 3 L SW (0.2 μm filtered). Three replicates (tanks) were set up per challenge group. Larvae received bacterial challenge via the rotifers.

3.2.8.2. Bacteria

3.2.8.2.1 *V. splendidus* DMC-1

One hundred ml cultures of MB were set up in baffled 250 ml conical flasks and inoculated with 1 colony of each bacterial strain. Cultures were shaken at 100 rpm for 24-h at 20 °C and the cells from 10ml of each culture harvested by centrifugation, washed twice in SSW and diluted to give 1×10^8 cells ml^{-1} ready to be added to rotifers at a 1/10 dilution to achieve a final concentration of 1×10^7 cells ml^{-1} in the rotifer suspension (see section 3.2.8.4). Again this was measured by OD_{600} and cell counts on MA plates.

3.2.8.3. Rotifer culture

About 2 million rotifers were collected from stock cultures at Seafish, Ardtoe, transported to the laboratory and transferred into sterile 10 L flasks containing 6 L

FSW with aeration. They were maintained on 1g Rotimac (Mariculture) per million rotifers per day. Rotimac was suspended in SSW and homogenised to break down large particles. Before addition to larval tanks, rotifers were enriched on *Pavlova* algae for 1 h prior to addition to tanks to feed larvae.

3.2.8.4. Colonisation of rotifers with bacteria

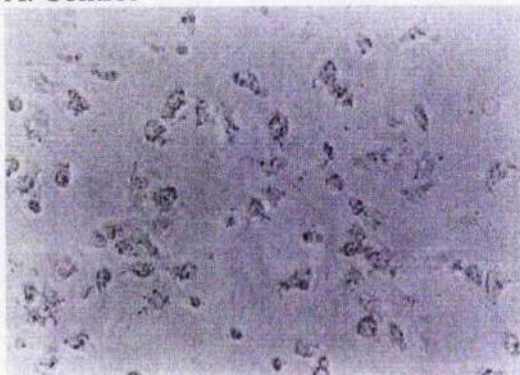
Rotifers were added on a daily basis throughout the challenge experiment at 1-2 ml⁻¹ on days 3-5 post-hatch and 4-6 ml⁻¹ thereafter. The challenge pathogen, *V. splendidus* DMC-1 was added on days 5, 6 and 7 post-hatch only. Rotifers were removed from their culture tanks and enriched with *Pavlova* algae (300 ml L⁻¹). They were siphoned, rinsed in SSW, concentrated to the required number ml⁻¹ and split into two groups: Control and DMC-1. Bacterial suspensions were added to rotifers for DMC-1 challenge group to a final concentration of 1 x 10⁷ cfu ml⁻¹ and were incubated for 1 h at room temperature. The final concentration of bacteria in the challenge tanks was 3 x 10⁴ cells ml⁻¹.

3.3. Results

3.3.1. Haemocyte assay

Haemocytes were visualised using an Olympus CK2 inverted microscope and cytotoxicity was measured by counting the proportions of rounded and spread cells in the wells of the assay plate; both types of cells are shown in Figure 3.2 from images recorded using a camera attachment to the microscope. The control (Figure 3.2 A) shows a high number of spread cells with few rounded cells. Control cells underwent transient rounding before returning to the spread configuration and the small proportion of rounded cells seen in these preparations was corrected for by normalising the results. The infected sample (Figure 3.2 B) shows the presence of the bacterial cells, and cytotoxic damage to the haemocytes can be seen as they begin to form a round shape (typical rounded cell is indicated with arrow in Figure 3.2). The positive control was *V. anguillarum* which was cytotoxic towards the haemocytes and the negative control was *Alteromonas* 1-1-1 which did not affect the haemocytes.

A. Control



B. Infected

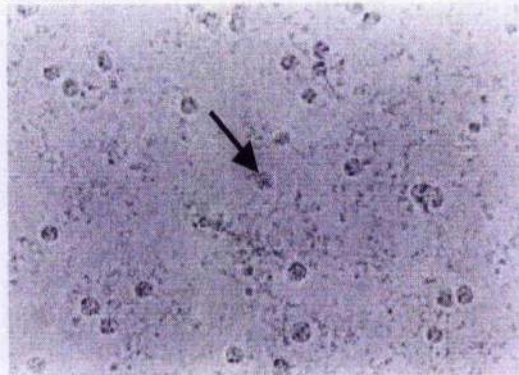


Figure 3.2. Control and bacteria treated *Mytilus edulis* haemocytes. Haemocytes were treated with *V. splendidus* as described in the text and photographs taken 1 h post-infection (Magnification 400x). The arrow indicates a typical rounded haemocyte.

A range of bacterial strains including *V. splendidus* biovars 1 and 2 and *V. alginolyticus*, were screened for cytotoxicity in the haemocyte assay (Appendix 1). These isolates were from the culture collection of strains isolated from turbot larvae and *Artemia* at Stolt Sea Farm marine hatcheries (Thomson, 2001). The washed bacterial cells of the pathogen of interest, DMC-1 were found to have medium cytotoxicity while the sterile supernatant was highly cytotoxic. Other isolates belonging to the *V. splendidus* biovar 1 group were screened; out of a total of 44, the overall cytotoxicity results were, high, 4 %; medium, 36 %; low, 55 % (Appendix 1). By testing both washed cells and filter-sterilised culture supernatants it was concluded that the toxic factor(s) were extracellular. Further work was carried out with sterile filtered culture supernatant to investigate the toxic factors of *V. splendidus* as this had a highly cytotoxic effect. Different dilutions of sterile supernatants from *V. splendidus* strains, DMC-1, LTS-3 and DTC-5 were screened in the haemocyte assay (Figure 3.3). The 24-h cultures of DMC-1 showed higher higher cytotoxicity than DTC-5 or LTS-3, with 50 % haemocyte rounding occurring at a dilution between 1/10 and 1/25, rather than between neat and 1/10. By 48-h the cytotoxic activity had increased in all three isolates, with both the DMC-1 and DTC-5 showing 50 % haemocyte rounding at a dilution between 1/25 and 1/50, whereas LTS-3 had only increased to between 1/10 and 1/25. As *V. splendidus* DMC-1 gave the highest cytotoxicity it was used to analyse cytotoxic activity in more detail.

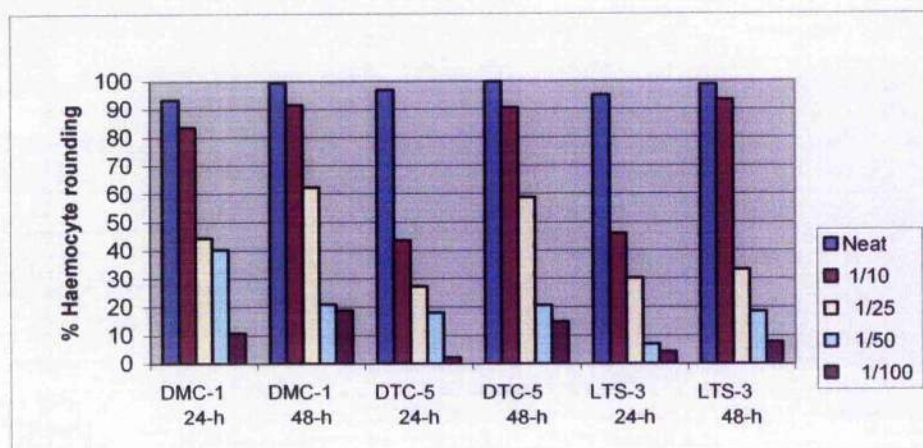


Figure 3.3. Haemocyte cytotoxicity assay of filter-sterilised culture supernatants of 3 *V. splendidus* strains DMC-1, DTC-5, LTS-3. Cytotoxic activity was titrated by treating mussel haemocytes with a dilution series (neat to 1/100) of 24 h and 48 h bacterial culture supernatants.

The cytotoxic activity was assessed after growing DMC-1 in two types of growth media, MB and TSB + 1.5 % NaCl and the effect of heating culture supernatant at 100 °C for 10 min was also assessed.

The effect of growth medium is illustrated in Figure 3.4. The native supernatants for both media gave similar cytotoxicity levels and with heat-treated supernatants (Figure 3.4 and 3.5), there was a marked reduction in cytotoxic activity, suggesting that the cytotoxic factor(s) is heat-labile. It was noted that there was a difference in susceptibility to heat between the two culture supernatants with the activity in TSB + NaCl being more resistant to heating at 100 °C than the MB. This suggests that there is possibly more than one cytotoxic factor being produced by this strain in the different growth media.

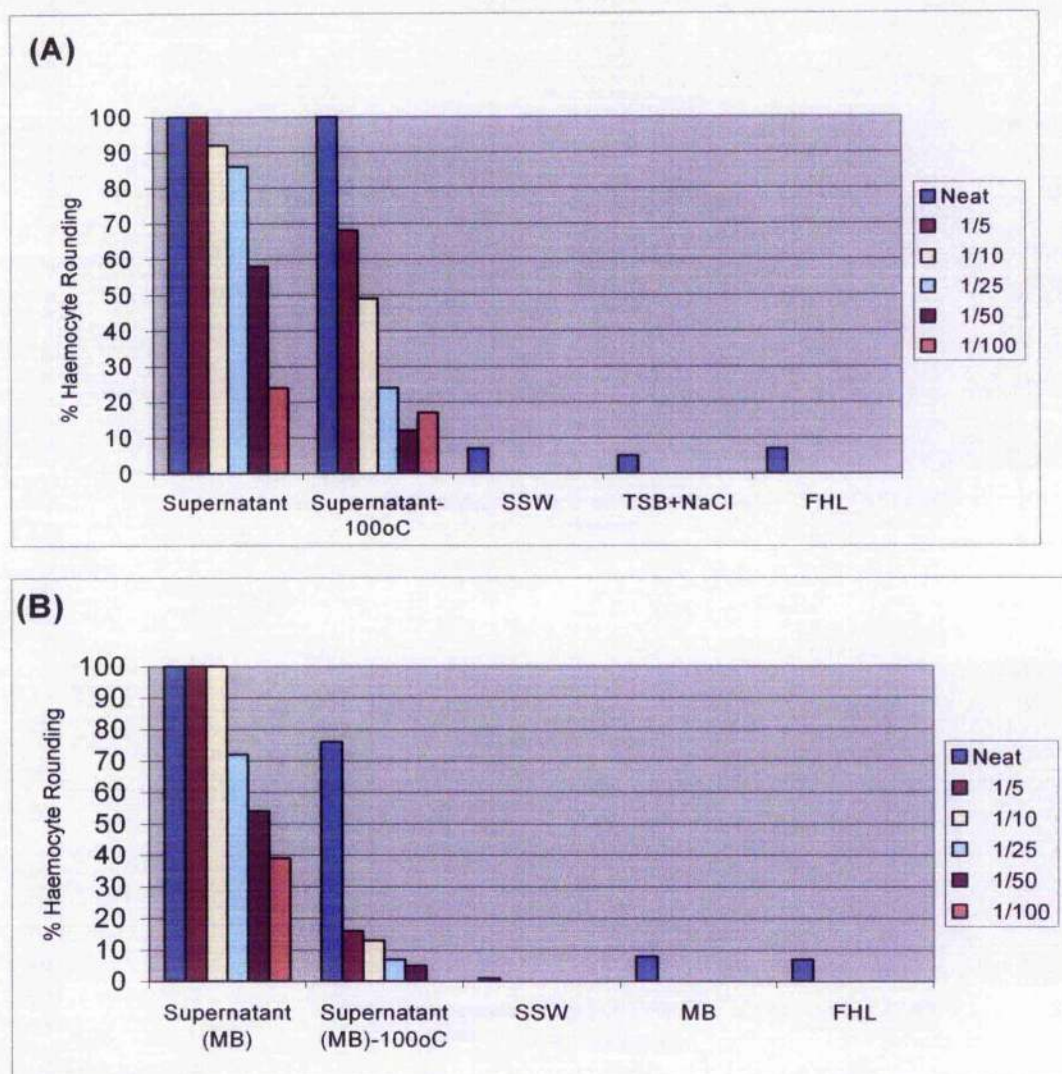


Figure 3.4. Haemocyte cytotoxicity assay of filter-sterilised supernatant of *V. splendidus* DMC-1. The bacteria was grown in **(A)** TSB + 1.5 % NaCl and **(B)** Marine broth (MB). The supernatant was applied to the haemocyte assay before and after heat treatment (100 °C x 10 min) at a range of dilutions (Neat-1/100). Negative controls were sterile seawater (SSW), Marine broth (MB) and filtered haemolymph (FHL).

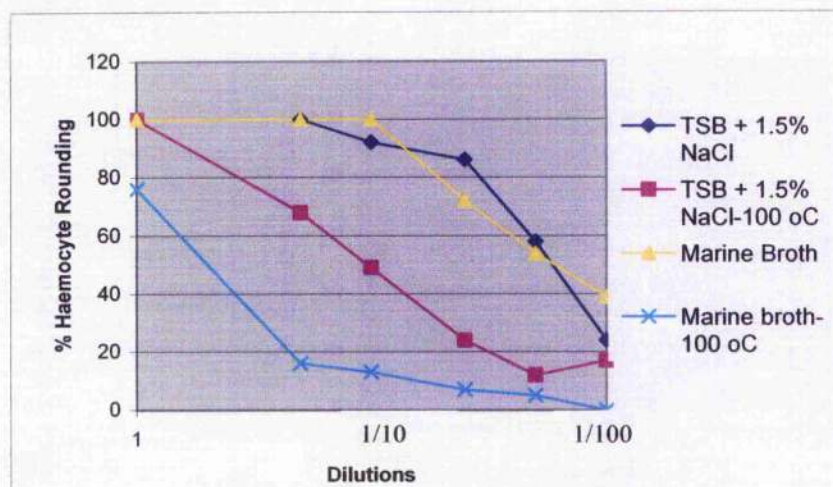
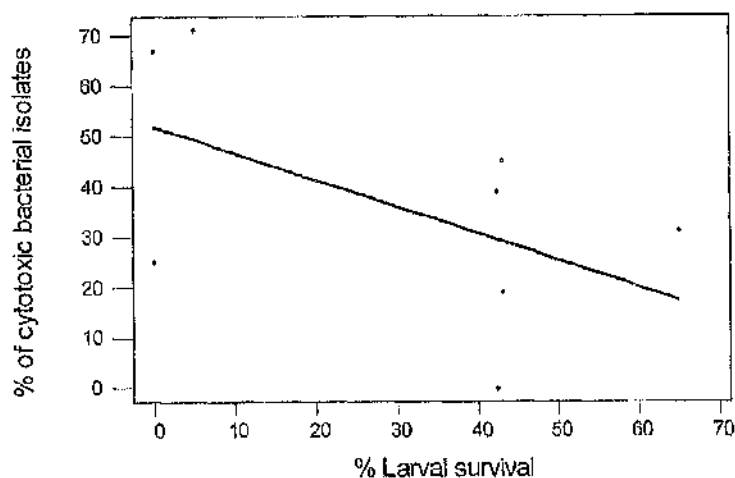


Figure 3.5. Haemocyte cytotoxicity assay with native and heat-treated (100 °C x 10 min) filter-sterilised culture supernatant of *V. splendidus* DMC-1 at dilutions neat-1/100. Bacterial cultures were grown in TSB + 1.5 % NaCl and Marine broth.

The haemocyte assay was originally developed and used to screen bacteria isolated from bivalve disease outbreaks (Lane and Birkbeck, 1999) but it was also used for isolates from turbot larvae, showing that poor larval rearing success was associated with the presence of a higher incidence of cytotoxic bacteria. In this study it was intended to screen bacteria from larval fish systems as a quick and easy *in vitro* assay to measure the cytotoxicity of bacterial strains before applying them to *in vivo* trials. However, comparison of cytotoxicity assay with data for virulence against turbot larvae (Thomson, 2001) (Table 3.1) showed that virulence did not always correlate with high cytotoxicity to haemocytes. Further evidence for this is shown in Figure 3.6 where correlation has been tested using Pearson correlation tests of the data from Table 3.1 and also data from work by Sinclair (1992) and Lane (1997). Both give negative correlation with p-values greater than 0.05 suggesting that there may be a relationship between *in vitro* and *in vivo* assays but it is not statistically significant.

Bacterial Strain	Turbot larval trial	% Haemocyte Rounding
DMC-1	Virulent	Medium
DTC-5	Virulent	High
HNF-3	Virulent	Low
HNF-8	Virulent	Low
LTH-1	Virulent	Medium
LTH-4	Virulent	Low
LTS-3	Virulent	High
DTC-2	Avirulent	Medium
DTR-2	Avirulent	Low
DTY-1	Avirulent	Medium
DTY-5	Avirulent	Medium
HNT-2	Avirulent	High
LTH-3	Avirulent	Low
LTS-4	Avirulent	Low
S3A2-11	Avirulent	Medium

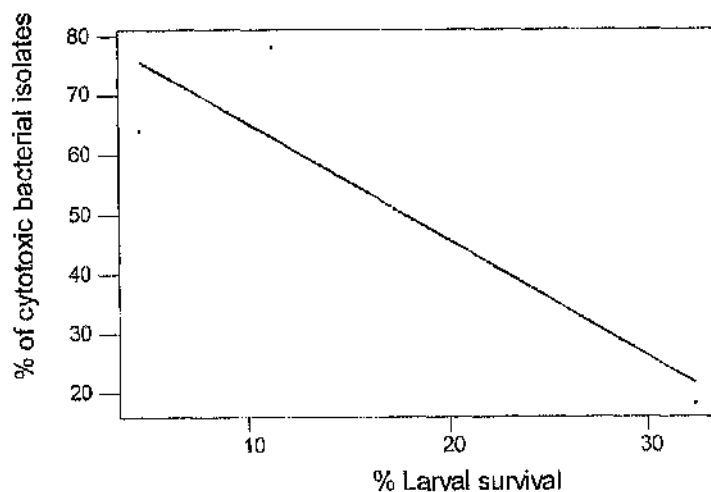
Table 3.1. Comparison of haemocyte cytotoxicity and larval toxicity of a selection of bacterial strains isolated from Stolt Seafarms, Spain. The virulence data was taken from Thomson (2001).



(Fish survival data from R. Thomson, MSc 2001)

Pearson correlation= -0.899

P-value= 0.289



(Data from P. Sinclair, PhD 1992 and E. Lane, PhD 1997)

Pearson correlation= -0.543

P-value= 0.131

Figure 3.6. Correlation between the percentage of turbot larvae surviving in production batches at commercial hatcheries and the percentage of bacterial isolates from larval samples which were classed as high or medium cytotoxicity in the haemocyte cytotoxicity assay.

3.3.2. Assay for haemolytic activity

The erythrocyte assay was used to screen for haemolytic and haemagglutinating activities on blood cells as both of these factors could play an important role in the pathogenicity of *V. splendidus*. From chapter 2 it was shown that by growing *V. splendidus* DMC-1 on blood agar, haemolysis could be detected (Figure 3.7). To quantify the amount of haemolysin produced and determine if the bacteria agglutinated the blood cells, erythrocyte assays were used with blood from different species.



Figure 3.7. Haemolytic activity of *V. splendidus* DMC-1 on blood marine agar.

Erythrocytes	Haemolysin titre of culture supernatant from		Haemagglutination titre of washed cells from	
	Marine Broth	TSB + 1.5 % NaCl Broth	Marine Broth	TSB + 1.5 % NaCl Broth
Turbot	>1/64	1/8	>1/64	1/16
Salmon	1/128	N.D.	1/16	N.D.
Sheep	1/256	1/2	1/4	-
Horse	1/128	-	-	-
Rabbit	1/256	1/2	-	-

Table 3.2. Haemolytic and haemagglutination activity of *V. splendidus* DMC-1. The activity of the bacterial supernatant grown in two growth media for 24 h was tested against a range of erythrocytes. N.D. Not tested, - not detected

With a range of erythrocytes it can be seen that haemolysin production was greater in MB than TSB + NaCl (Table 3.2). Later experiments showed that haemolysin was

produced at a slower rate in TSB + NaCl to a lower level than in MB (data in Appendix 10). Table 3.2 also shows that the haemolysin has a broad-spectrum of activity with similar titres for all blood types tested.

Further studies on the haemolysin showed that the haemolytic activity of *V. splendidus* DMC-1 supernatant against sheep erythrocytes was destroyed by heating at 100 °C for 10 min, reducing the haemolysin titre from 1/128-1/256 to <1/2 (Figure 3.8).

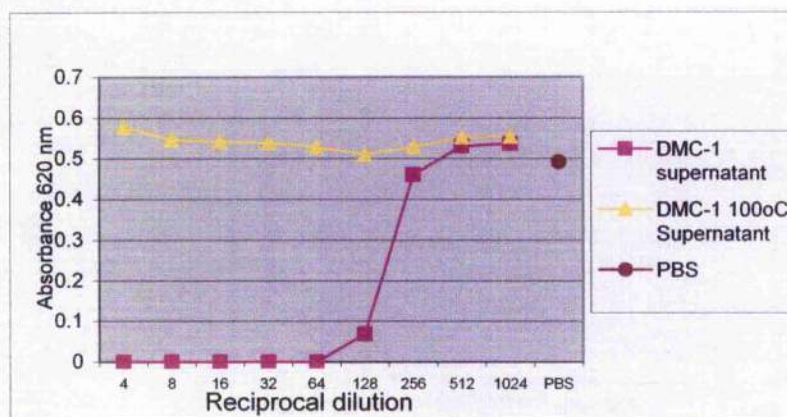


Figure 3.8. Haemolytic activity of *V. splendidus* DMC-1 supernatant. The haemolytic activity was measured using cell scattering measurements at an absorbance of 620 nm. The haemolytic activity was recorded by a reduction in absorbance. Native bacterial supernatant and heat-treated supernatant (100 °C x 10 min) were both tested for haemolytic activity. PBS was used as a negative control.

Expression of haemagglutinin was similarly affected by growth medium (Table 3.2) but appeared to be specific for turbot cells.

3.3.3. Turbot macrophage assays

3.3.3.1. Cytotoxicity assay

As the mussel haemocyte assay results did not correlate with bacterial virulence in larval turbot, it was decided to test turbot macrophages in a cytotoxic assay as they represented a more relevant target cell. However, it proved difficult to separate the blood cells from the macrophage and the supply of turbot erythrocytes was limited. Therefore, the use of turbot macrophage in these assays was not an easy alternative to the haemocyte assay and further alternative cell assays were investigated.

3.3.3.2. Tissue Culture

3.3.8.2.1 TV1-S4

The bacterial culture supernatant concentrated 100-fold using a tangential flow system had a titre of $1/1024$ HU ml^{-1} and was applied to TV1-S4 tissue culture cells at a range of dilutions. The *V. splendidus* DMC-1 culture supernatant was highly cytotoxic to TV1-S4 cells as measured in an XTT assay (Figure 3.9) or when assessed visually with an inverted microscope (Figure 3.10). The control cells are arranged in an even monolayer, however, addition of the bacterial supernatant disrupted the monolayer with the cells detaching from the surface of the plate and leaving clusters of damaged cells that had lost their integrity. The dilution of $1/160$ still caused damage with “plaques” being visible in comparison to the control monolayer. These results showed that the *V. splendidus* extracellular material with a high haemolytic titre was cytotoxic towards cells of turbot origin. Therefore, turbot tissue culture cell lines provide a more appropriate *in vitro* assay to assess the cytotoxicity of bacterial strains.

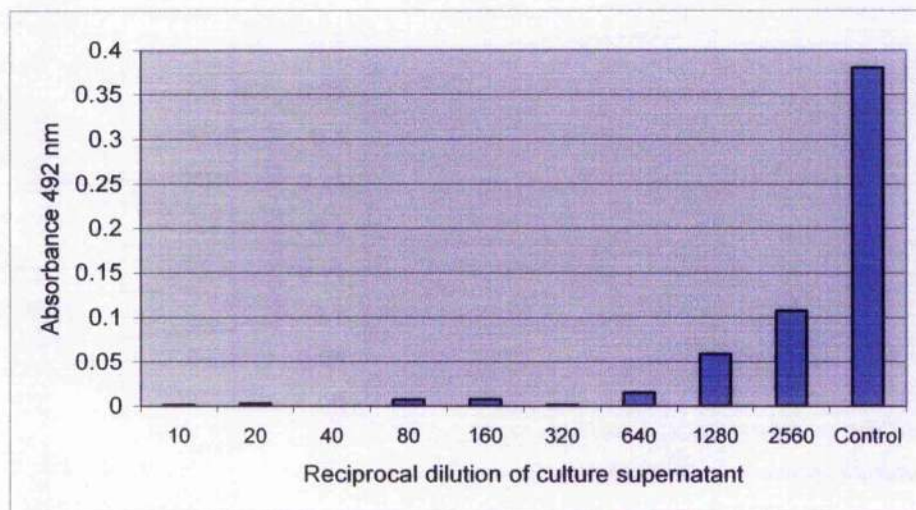


Figure 3.9. Effect of concentrated *V. splendidus* culture supernatant on TV1-S4 turbot cells measured in an XTT assay. The concentrated *V. splendidus* DMC-1 supernatant was applied to tissue culture monolayers in a 24-well plate at a range of dilutions ($1/10$ - $1/2560$) and was incubated for 24 h at 20°C . The level of cell destruction was measured using a colorimetric assay based on conversion of XTT to a coloured formazan product measured on a plate spectrophotometer at 492 nm.

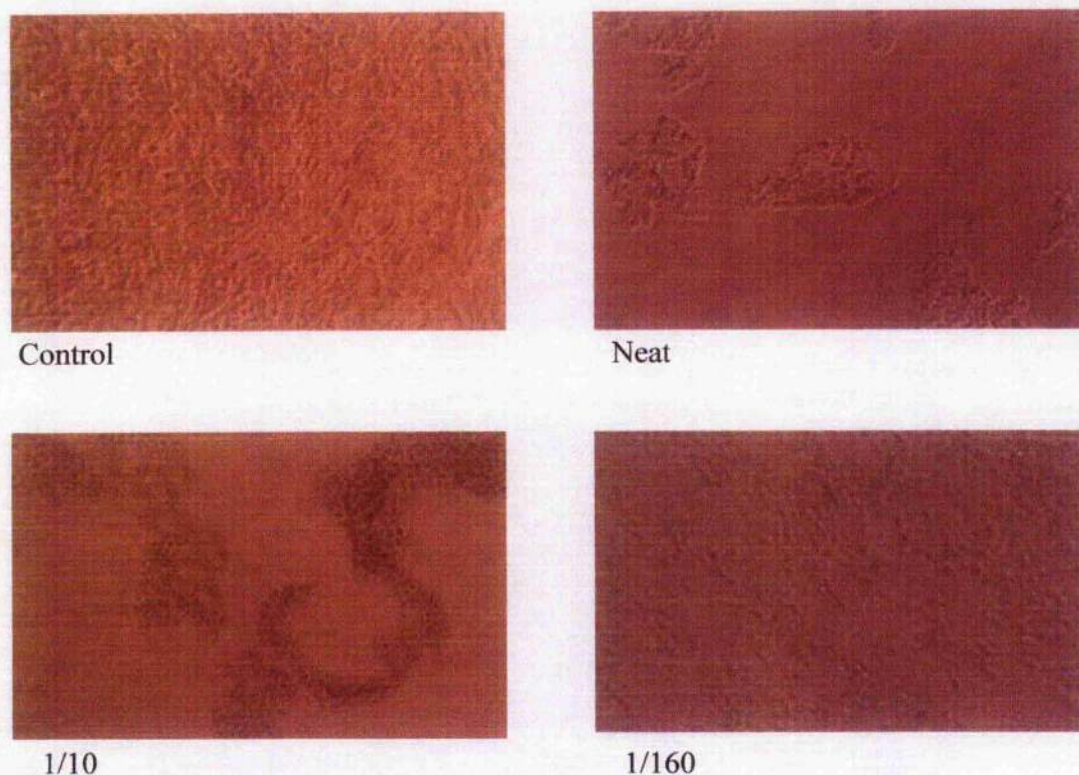


Figure 3.10. TV1-S4 turbot cells treated with concentrated *V. splendidus* DMC-1 culture supernatant at dilutions neat, 1/10, 1/160. The negative control contained PBS. The TV1-S4 cells were assessed visually using an inverted microscope (Magnification x400).

3.3.4. Yolk-sac larval trials 1 & 2-Bergen

When yolk-sac turbot larvae were challenged with *V. splendidus* DMC-1 in two experiments mortalities were similar to those in control groups showing that *V. splendidus* DMC-1 was not pathogenic towards larvae at this stage of growth (Figure 3.11). There was no significant difference between the control groups and those exposed to bacteria (Chi-square test).

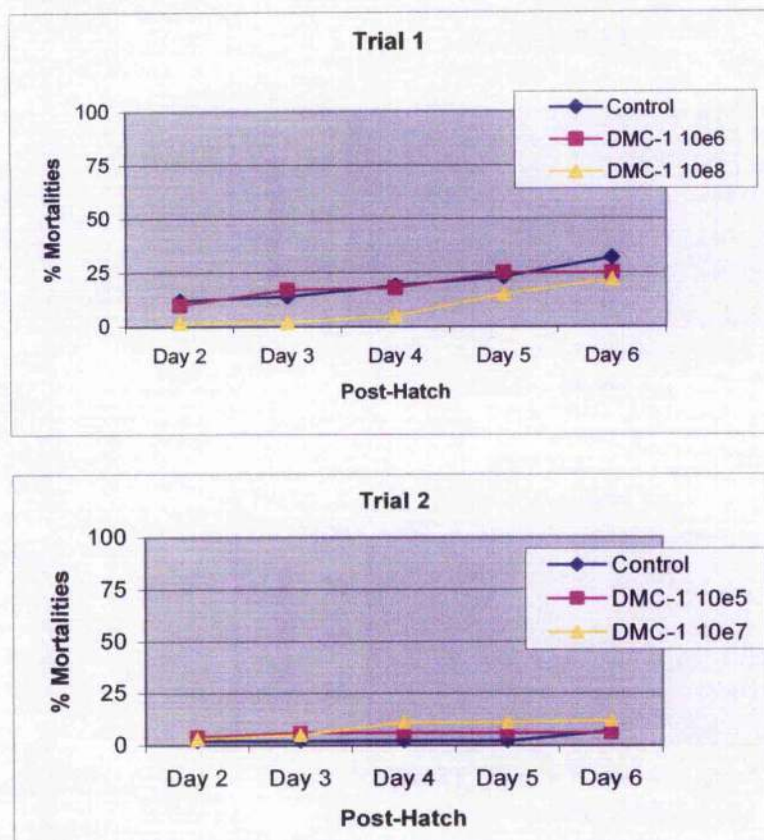


Figure 3.11. Turbot yolk-sac larval challenge trials 1 and 2. Eggs were distributed individually into 2 ml SSW in 24-well tissue culture plates where they hatched as yolk-sac larvae. The challenge groups received 100 μ l of *V. splendidus* DMC-1 at final concentrations in trial 1 of 1×10^6 and 1×10^8 cfu ml⁻¹; trial 2, 1×10^5 and 1×10^7 cfu ml⁻¹, directly after distribution of the eggs into the wells of the tissue culture plate. Trials were carried out in challenge facilities at IMR, Bergen, Norway.

3.3.5. First-feeding turbot larval challenge trial-Bergen

As *V. splendidus* DMC-1 was isolated from the intestinal tract of first-feeding turbot larvae, it was decided to test the effect of the organism on larvae of this developmental stage. The results from the first-feeding trial at IMR, Bergen showed that *V. splendidus* DMC-1 was virulent towards first-feeding larvae (Figure 3.12). Only one control tank was available due to the second tank of larvae crashing 24 h after the experiment was set up. Nevertheless, it is clear from the results in Figure 3.12 that after addition of *V. splendidus* DMC-1 on days 4 and 5, both tanks experienced high mortalities. Statistically, these mortalities were significantly different from the mortalities in the control tank (Table 3.3). The control group mortalities showed an increase on days 8 and 9 at the end of the experiment, which

can occur at the marine hatcheries due to the presence of bacteria in the system from external sources.

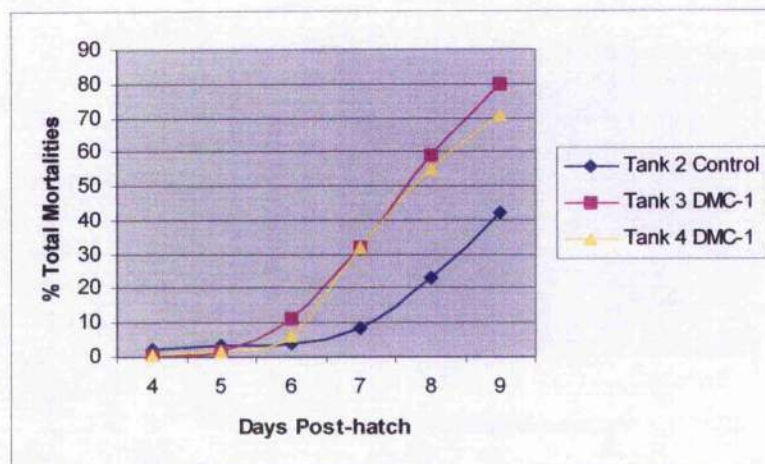


Figure 3.12. First-feeding turbot larval challenge. Turbot larvae were held in 160 L tanks in 45 L of SW (1500 larvae per tank). The challenge groups received *V. splendidus* DMC-1 via the live food of rotifers on days 4 and 5. The control group was fed rotifers alone. Trial was carried out in challenge facilities at IMR, Bergen.

Groups	p-value		Significantly different	
	Day 8	Day 9	Day 8	Day 9
DMC-1 Tank 3	0.000	0.000	Yes	Yes
DMC-1 Tank 4	0.000	0.000	Yes	Yes

Table 3.3. Chi-square test of larval mortality data from first-feeding trial in Bergen of control versus challenge group, *V. splendidus* DMC-1. The % mortalities for the control and the challenge groups were used to analyse the differences between the groups using Chi-square analysis. p-value <0.001= highly significant.

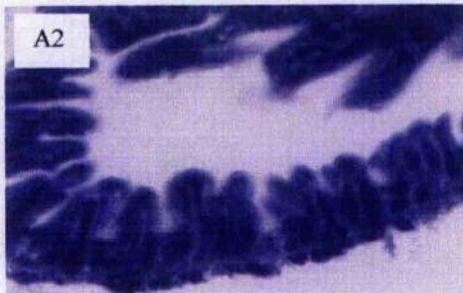
3.3.5.1. Immunohistochemistry

Samples of active larvae were taken throughout the challenge experiment to determine if damage occurred in the gut of the larvae infected by *V. splendidus* DMC-1. Larvae that were sampled were processed and analysed using immunohistochemistry at IMR, Bergen. Analysis of sections of a number of larvae showed a high number of bacteria within the gut of the larvae. The main location of

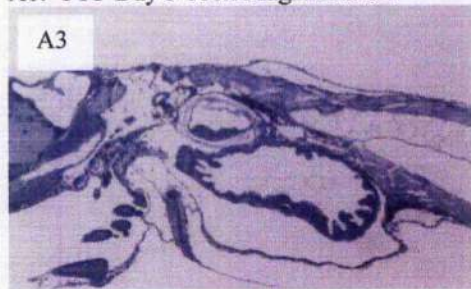
V. splendidus was the intestinal epithelium and enteritis was typically seen in infected larvae. Large amounts of positively stained (red) bacteria were found in the intestinal lumen (Figure 3.13, B1-B6), with strong positive staining along the intestinal epithelium. In several sections, indications of damage to the intestinal epithelium were found (Figure 3.13, arrow 6), indicating probable enteritis. In moribund larvae there was signs of septicaemia with positive staining of bacteria in the kidney. In control larvae, fed "normal" rotifers without bacterial enrichment no sign of enteritis was evident (Figure 3.13, A1-A4). The exception to this pattern was found at day 9, when all three control larvae had large amounts of positively-stained bacteria in the gastrointestinal tract (not shown). There was no evidence that *V. splendidus* DMC-1 infected other areas of the larvae, apart from in moribund larvae, therefore providing evidence that this pathogen initially has specificity for the gut.



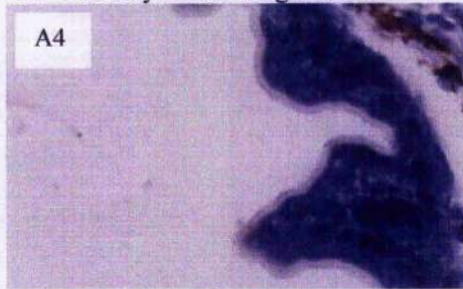
A1. 1 FF Day 5 100x Magnification.



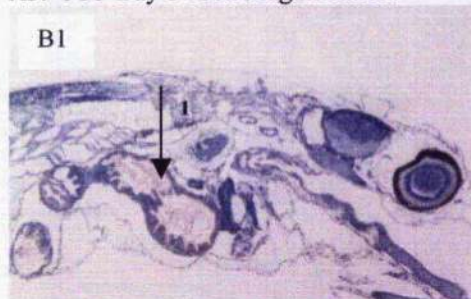
A2. 1 FF Day 5 400x Magnification



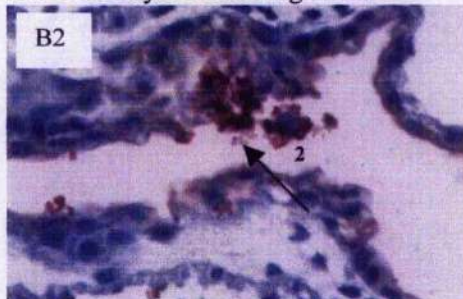
A3. 1 FF Day 8 100x Magnification.



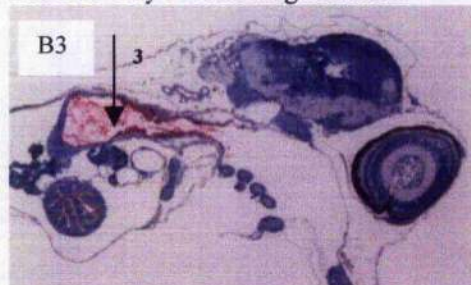
A4. 1 FF Day 8 1000x Magnification.



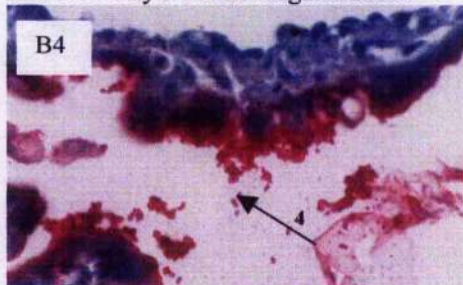
B1. 2 FF Day 5 100x Magnification.



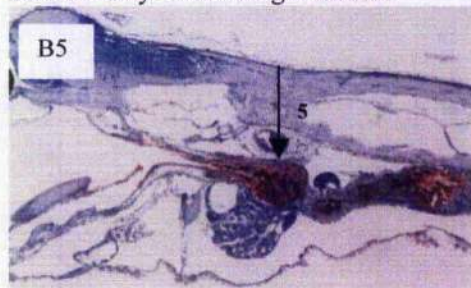
B2. 2 FF Day 5 1000x Magnification.



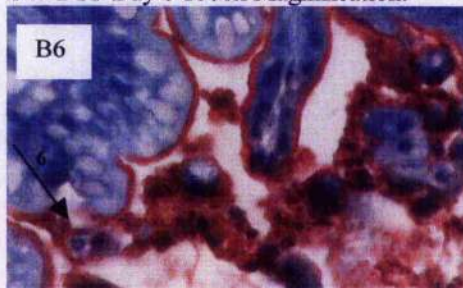
B3. 2 FF Day 8 100x Magnification.



B4. 2 FF Day 8 1000x Magnification.



B5. 2 FF Day 9 100x Magnification.



B6. 2 FF Day 9 1000x Magnification.

Figure 3.13. Immunohistochemistry slides of larval turbot sections from first-feeding trial at IMR, Bergen. A1-A4, control (1FF) and B1-B6, DMC-1 (2FF) challenged first-feeding (FF) larvae sampled days 5, 8 and 9. **Arrows 1, 3 and 5** Positively stained bacteria in the larval gut; **2 and 4** Individual positive stained bacteria; **6** Damage to the brush border of the gut epithelium.

3.3.6. First-feeding larval challenge trial-Glasgow

A first-feeding trial was carried out using a scaled-down version of the trial set up in Bergen. The results from this trial showed high mortalities in the challenge group DMC-1 (Figure 3.14), but mortalities in the DMC-1 group were only significantly higher than those in the control group on day 9 and not by day 10 (Table 3.4). The high mortalities recorded in the control group were skewed by the occurrence of mortalities in one of the three tanks in that group (Figure 3.15). The % survivals in Figure 3.15 show the exact end point of survivals for each of the tanks showing that the control survivals were widely spread. This confirmed earlier data of Thomson (2001) that *V. splendidus* DMC-1 was virulent for turbot larvae when administered via rotifers. Additional trials using a higher number of group replicates were carried out however, overall survival of the larvae was poor as a result of the extensive transport time from the hatchery to the laboratory during winter months.

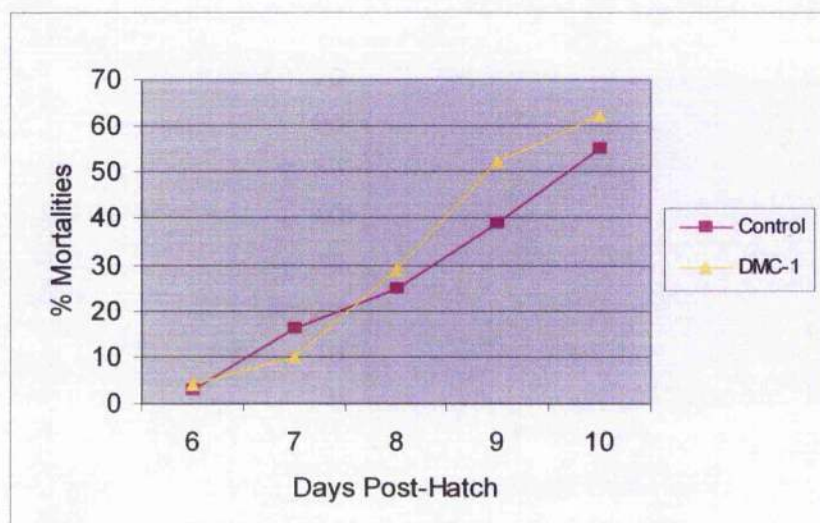


Figure 3.14. First-feeding turbot larval challenge. Trial was carried out in a controlled environment room in the laboratory in Glasgow using 5 L flasks containing 50 larvae in 3 L of SSW with three replicates per challenge group. Larvae were challenged with *V. splendidus* DMC-1 on days 5-7.

Groups	p-value		Significantly different	
	Day 9	Day 10	Day 9	Day 10
Control vs. DMC-1	0.028	0.241	Yes	No

Table 3.4. Chi-square of mortality data from first-feeding trial in Glasgow of control versus challenge group *V. splendidus* DMC-1.

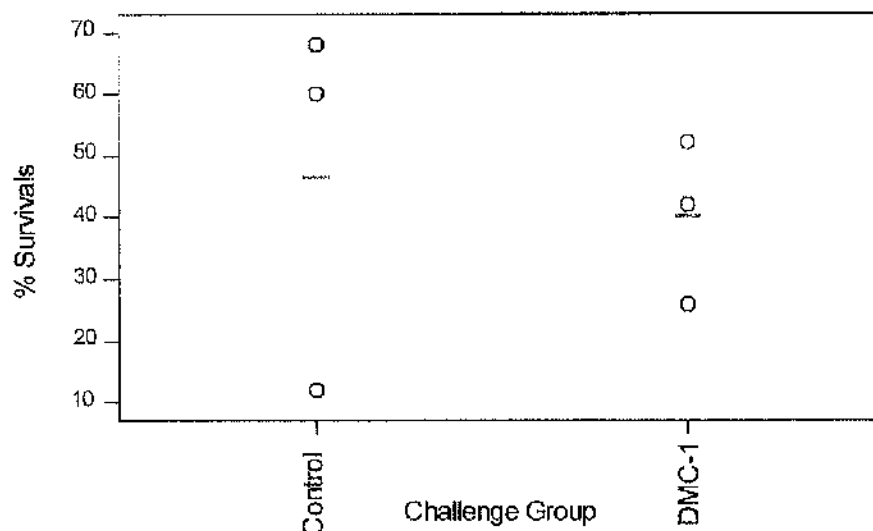


Figure 3.15. Larval survival data of first-feeding trial in Glasgow on day 10 post-hatch (Figure 3.14). The larvae received rotifers on a daily basis and the challenge group were given *V. splendidus* DMC-1 on day 5-7 along with the rotifers. The group means are indicated by the horizontal lines.

3.4. Discussion

Although the cells and culture supernatants from several bacteria that were pathogenic towards larval turbot were cytotoxic in the haemocyte assay (Thomson, 2001), other bacterial strains did not show a good correlation between haemocyte assay and larval virulence trials (Figure 3.6). Therefore, other *in vitro* assays were sought that might correlate with virulence and these included erythrocyte assays with a range of blood types, turbot macrophage assays and tissue culture assays with fish cell lines. In this way more appropriate *in vitro* assays for screening bacteria isolated from turbot, were established.

The *in vivo* trials showed that *V. splendidus* DMC-1 was pathogenic towards first-feeding turbot larvae but not at the yolk-sac stage. Larvae at this stage use their endogenous yolk-sac and the only entry routes for pathogens is via the water (through drinking) or by invasion, as probably occurs for *V. anguillarum*. Given the very low quantities of water taken up by drinking, few bacteria would be ingested by this route. According to studies by Rietan *et al.* (1998), less than 3 % of bacterial

uptake is via the water. Bacteria within the gut of larvae are mainly obtained through the ingestion of live food (Munro *et al.*, 1993; 1994), and this would explain why there were no pathogenic effects at the yolk-sac larval stage.

In the first-feeding trials *V. splendidus* DMC-1 caused high mortalities. This together with the results from the yolk-sac trials suggests that DMC-1 requires entry into the gut of the larvae for pathogenic effects to occur and that DMC-1 is a specific gut pathogen. As the bacteria were originally isolated from the gut of first feeding larvae, it is not surprising that the pathogenic effects were only seen in the first feeding larval trials when the larvae had ingested live food containing high numbers of bacteria.

Immunohistochemistry revealed large amounts of bacteria in the intestines of challenged larvae. Larvae challenged with *V. splendidus* DMC-1 showed typical signs of enteritis with damage to the brush border and positively stained bacteria interstitially. Moribund larvae showed signs of septicaemia, with positively stained bacteria in the kidney.

Therefore, the overall results from the *in vivo* trials have shown that *V. splendidus* DMC-1 caused high mortalities when added to first-feeding turbot larval systems. Also confirmed was that DMC-1 is a gut pathogen of turbot larvae. These pathogenic effects of *V. splendidus* strains were also shown by the work of Gatesoupe *et al.* (1999).

The *in vitro* trials identified cytotoxic/haemolytic factor(s) present in the bacterial supernatant that may be one of the virulence factors of this strain that is responsible for the enteritis in the larval gut. Further work involved investigating the pathogenic mechanisms and if this cytotoxic factor is involved in the damage to the larval gut and the subsequent killing of the larvae.

Chapter 4. Identification of Potential Virulence Factors of *Vibrio splendidus* DMC-1.

4.1. Introduction

Pathogenic vibrios produce numerous virulence factors including enterotoxins, haemolysins, cytotoxins, proteases, adhesins or haemagglutinins, and siderophores, most of which are proteinaceous. The most important virulence factor produced by many vibrios is an enterotoxin, with cholera enterotoxin being one of the most extensively studied bacterial protein toxins. Haemolysins have been widely studied in vibrios, and have been found to play a role in virulence in a range of pathogenic fish vibrios, including *V. anguillarum* (Toranzo *et al.*, 1983) and *V. damsela* (Fouz *et al.*, 1993). This chapter focuses on identifying some potential virulence factors associated with *V. splendidus* DMC-1.

4.2. Materials and Methods

4.2.1. Protein precipitation using Ammonium sulphate

One hundred ml volumes from 24 h TSB + 1.5 % NaCl cultures of *V. splendidus* DMC-1, DTC-5 and LTS-3 were centrifuged at 5,000 x g x 10 min. Culture supernatant was dispensed in 20 ml volumes and solid ammonium sulphate was added, to give 30 %, 50 %, 70 % and 90 % saturation according to Green and Hughes (1955). The mixtures were incubated at 20 °C for 4 h with gentle agitation and centrifuged at 5,000 x g x 10 min to collect the precipitates. All traces of liquid were removed from the pellets by inverting tubes. Pellets were stored overnight at 4 °C or used immediately. The pellets were resuspended in 4 ml of SSW (to give a 5-fold concentrate) and dialysed overnight against SSW. Levels of cytotoxic factors were tested in the haemocyte assay (Section 3.2.1), to assess the best level of ammonium sulphate saturation to use for further precipitation work.

4.2.2. SDS-PAGE

Precast gels and a NuPAGE Mini-Cell gel apparatus were from Novex (Invitrogen). Samples and buffers were prepared according to the manufacturer's instruction with NuPAGE 12 % Bis-Tris electrophoresis gels (Appendix 8 and 9). Volumes of 10 µl of samples and Mark 12 standard marker proteins were applied to 12 % Bis-Tris gels with MOPS as running buffer. The gels were run at 200 V (constant voltage) for 50 min, and the gels then removed from the cassettes and fixed for 10 min at room

temperature. The gels were stained for 1 h or overnight in a 1/10 dilution of Coomassie Brilliant Blue, and destained until excess colour was removed.

4.2.3. Isoelectric focusing

A 1 L TSB + 1.5 % NaCl 24 h culture was used to concentrate the cytotoxic factors in the supernatant by ammonium sulphate precipitation (70 % saturation). The pellet was resuspended in 40 ml 1 % glycine and added to pre-soaked (1 % glycine) dialysis tubing (10 kD cut-off) and dialysed against 4 L of 1 % glycine at 4 °C. The glycine solution was replaced with fresh solution after 8 h and the material was left to dialyse overnight. The contents of the dialysis tubing were added to a beaker containing 5 ml ampholines (Pharmacia), and the volume was made up to 100 ml with distilled water (dH₂O). Sephadex G-200 (Pharmacia) (3.5 g) was added slowly to the solution, which was held on ice and stirred constantly with a glass rod until a smooth paste was obtained. The gel was poured into the mould of the IEF plate, ensuring that the gel was spread evenly. Six electrode strips were soaked in a 1/20 solution of ampholines and 3 strips were applied to each end of the gel. One strip soaked in 0.1 M NaOH was added to the cathode and 1 strip in 0.1 M H₃PO₄ was applied to the anode. Isoelectric focusing was carried out overnight at constant power (8 watts) at 1 °C.

After focusing the gel was divided into 36 fractions using the metal grid provided; fractions were removed carefully and added to filter units to remove sample from the Sephadex. A 5 ml volume of saline was added to each column and eluted proteins were collected in universal bottles. The samples were kept on ice at all times. The pH, protein concentration and haemocyte cytotoxicity of all fractions were measured. The pH of each fraction was measured using a pH meter calibrated before use using pH standards. The protein concentration was measured using the Bradford assay (see 4.2.4 microtitre plate Bradford assay), and cytotoxicity was measured using the haemocyte assay (section 4.2.5).

4.2.4. Microtitre plate Bradford assay

Bovine Serum Albumin (BSA) standards ranging from 0-2000 µg ml⁻¹ were prepared in saline, to provide a range of protein standards. For the working range 100 to 1,500 µg ml⁻¹, 5 µl of each standard or unknown sample was pipetted into the appropriate

wells of a microtitre plate. A 5 μ l volume of diluent was used for the blank wells, and 200 μ l of the Coomassie Reagent (PIERCE) was added to each well. For the working range 1 to 25 μ g/ml, 150 μ l of standards or unknown sample was pipetted into wells and 150 μ l of Coomassie Reagent was added. The absorbance was measured at 570 nm on an Athos plate reader.

4.2.5. Haemocyte assay of IEF fractions

The haemocyte assay was carried out as before (section 3.2.1.). A 200 μ l volume of each fraction from IEF was applied to the wells of the assay plate, initially as pooled groups of 5 fractions at three concentrations, neat, 10^{-1} and 10^{-2} . For those pooled fractions found to be cytotoxic, the fractions were then tested in groups of 2. The positive controls included 200 μ l of normal DMC-1 supernatant, the starting ammonium sulphate concentrate and dialysed DMC-1 supernatant, and the negative control in the assay was SSW. The samples were tested in duplicate.

4.2.6. Electrophoretic transfer to PVDF membrane

The NuPAGE system was used to electrophorese 25 μ l samples in SDS-PAGE gels before transfer to PVDF membranes. Details of buffers are given in Appendix 9. Blotting pads were saturated in transfer buffer, and air bubbles removed by squeezing the pads whilst they were submerged in buffer to ensure there was no block in transfer of proteins. PVDF (proBlott) transfer membrane and filter paper were cut to the dimensions of the gel and filter paper was soaked in transfer buffer prior to use. One half of the electrophoresed gel cassette was removed, leaving the gel resting on the larger plate. The upper part of the gel containing the wells was removed with a knife and on removal of the gel from the plate the "foot" of the gel was removed prior to soaking in transfer buffer for 5 min. The gel was placed onto pre-soaked filter paper and the surface of gel moistened with transfer buffer. A transfer membrane was pre-wetted in methanol and rinsed in transfer buffer prior to placing onto the gel surface, ensuring that all air bubbles were removed. Pre-soaked filter paper was placed on the top. The blotting module was prepared by placing 2 pre-soaked blotting pads into the cathode core of the blot module and the gel membrane assembly was placed on top of these pads with the gel closest to the cathode plate. A further four blotting pads were added on top of the gel membrane so that they rose 0.5 cm over the top of cathode

core. The anode core was placed on top of the assembly to hold the gel/membrane securely in place and the blot module was placed into the Mini-Cell apparatus, and a tight fit was ensured. The blot module was filled with transfer buffer until the gel/membrane sandwich was fully submerged. The outer chamber was filled with MilliQ ultrapure distilled water and the blotting system was run overnight at 30 V with a cooling system attached to maintain the temperature at 4 °C.

The blot membrane was carefully removed from the sandwich, rinsed with MilliQ ultrapure water and dipped in methanol prior to staining. The membrane was stained in concentrated Coomassie Brilliant Blue stain for 1 min, destained in 50 % v/v methanol and washed in MilliQ ultrapure water. After allowing the membrane to dry the major protein band was excised and retained for sequencing.

4.2.7. N-terminal Sequencing

The Molecular Palaeontology Laboratory in the Department of Geology and Applied Geology, University of Glasgow carried out the protein sequencing and amino acid analyses, using an ABI 477A pulsed-liquid protein sequencer and an ABI 420H amino acid analyser. The protein sequencing and amino acid analysis was carried out on protein bound to PVDF as described above. After sequencing, a computer printout for each chromatographic cycle and a computer interpretation of the sequence was provided. This was checked by manual examination of raw data. The sequence was entered into the computer on the website for the European Bioinformatics Institute (EBI) database and analysed using FASTA to compare against other amino acid sequences.

4.2.8. Zymogram gel

Bacterial cultures grown in MB for 24 h were harvested by centrifugation and the supernatant was filter-sterilised (0.22 μm). One volume of filter-sterilised supernatant was mixed with an equal volume of Novex Tris-Glycine SDS sample buffer (2x) and incubated at room temperature for 10 min. The samples were loaded onto a Zymogram (blue casein, 4-16 % Tris-Glycine) gel (10-25 μl) and electrophoresed in a Novex Mini-Cell system with 1x Tris-glycine SDS Running buffer. Electrophoresis was at 125 V constant for 90 min. The gel was removed from the cassette with a gel knife and placed in renaturing buffer (2.5 % Triton X-100 in dH₂O) with agitation at room temperature for 30 min. The buffer was replaced with 0.1 M glycine (pH 7.5) and agitated at room temperature for 30 min. The buffer was replaced for a second time with fresh 0.1 M glycine and incubated at 37 °C for at least 4 h. The gel was incubated overnight for maximum sensitivity. Areas of protease activity were shown as clear zones.

4.2.9. Protease assay

The protease assays were carried out as in section 2.2.10, with the substrates azocoll, azocasein and elastin-Congo red.

4.2.10. Caseinase production on milk agar plates

Marine agar plates were prepared containing sterile 4 % skimmed milk (Marvel). After inoculation, plates were incubated at 20 °C for 3 days; a positive reaction was shown by the presence of clear zones around the bacterial colonies.

4.2.11. Phospholipase production on egg-yolk agar

This was carried out according to section 2.2.9.

4.2.12. Haemolysin assays

This was carried out in accordance with section 2.2.8 and 3.2.2.

4.3. Results

The production of extracellular proteins that could contribute to virulence was studied initially with the bacterial supernatant of *V. splendidus* DMC-1. To concentrate bacterial proteins in the culture supernatant, ammonium sulphate precipitation was used. In initial experiments concentrations of varying degrees of saturation (30, 50, 70 and 90 %) were used and the precipitated fraction redissolved and tested at 1/10 and 1/100 dilutions in the haemocyte assay for cytotoxicity. From the results shown in Figure 4.1 the 70 % and 90 % saturated solutions gave the highest yield of cytotoxic factor(s) and 70 % saturation was chosen for future concentration of culture supernatants.

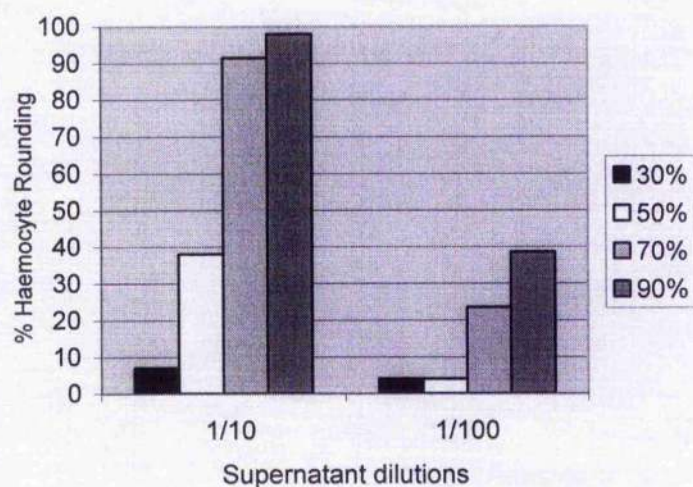


Figure 4.1. Ammonium sulphate ($(\text{NH}_4)_2\text{SO}_4$) precipitation of cytotoxic factor(s) from DMC-1 TSA + 1.5 % NaCl supernatant. Precipitated protein at various degrees of ammonium sulphate saturation (30-90 %) were redissolved and the cytotoxic activity of the fractions measured at 1/10 and 1/100 dilutions in the haemocyte cytotoxicity assay.

4.3.1. Analysis of culture supernatants by SDS-PAGE

The supernatants from *V. splendidus* DMC-1 cultures grown for 24 h in TSB + 1.5 % NaCl were analysed by SDS-PAGE gels both before and after concentration of the protein by ammonium sulphate precipitation. Figure 4.2 shows the protein banding patterns of both native and concentrated DMC-1 supernatant. A dominant band of 40 kD was found in both supernatants, but other weaker bands were only seen once the supernatant had been concentrated.

The culture supernatants of two strains of *V. splendidus* biovar 1, DMC-1, which is virulent and DTC-2 which is avirulent (Thomson, 2001), were compared by SDS-PAGE. Figure 4.3 shows results for both culture supernatants sampled at 16 and 24 h and after concentration with $(\text{NH}_4)_2 \text{SO}_4$. The intensity of protein bands was greater with the older cultures and after concentration of the supernatants many minor protein bands were apparent. No clear differences were seen that would be correlated with the difference in virulence of these two strains and the dominant band in both strains was at 40 kD.

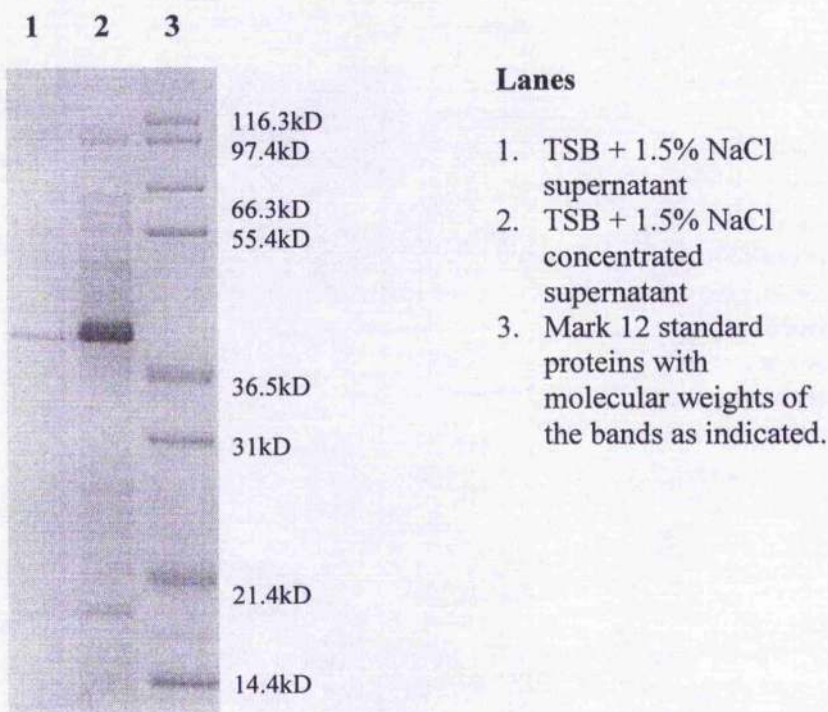


Figure 4.2. Analysis of protein in *V. splendidus* culture supernatant from cultures grown in TSB + 1.5 % NaCl using SDS-PAGE gels. Protein standards are listed at

the side in kD using Mark12 marker. The gel was stained with Coomassie Brilliant Blue stain.

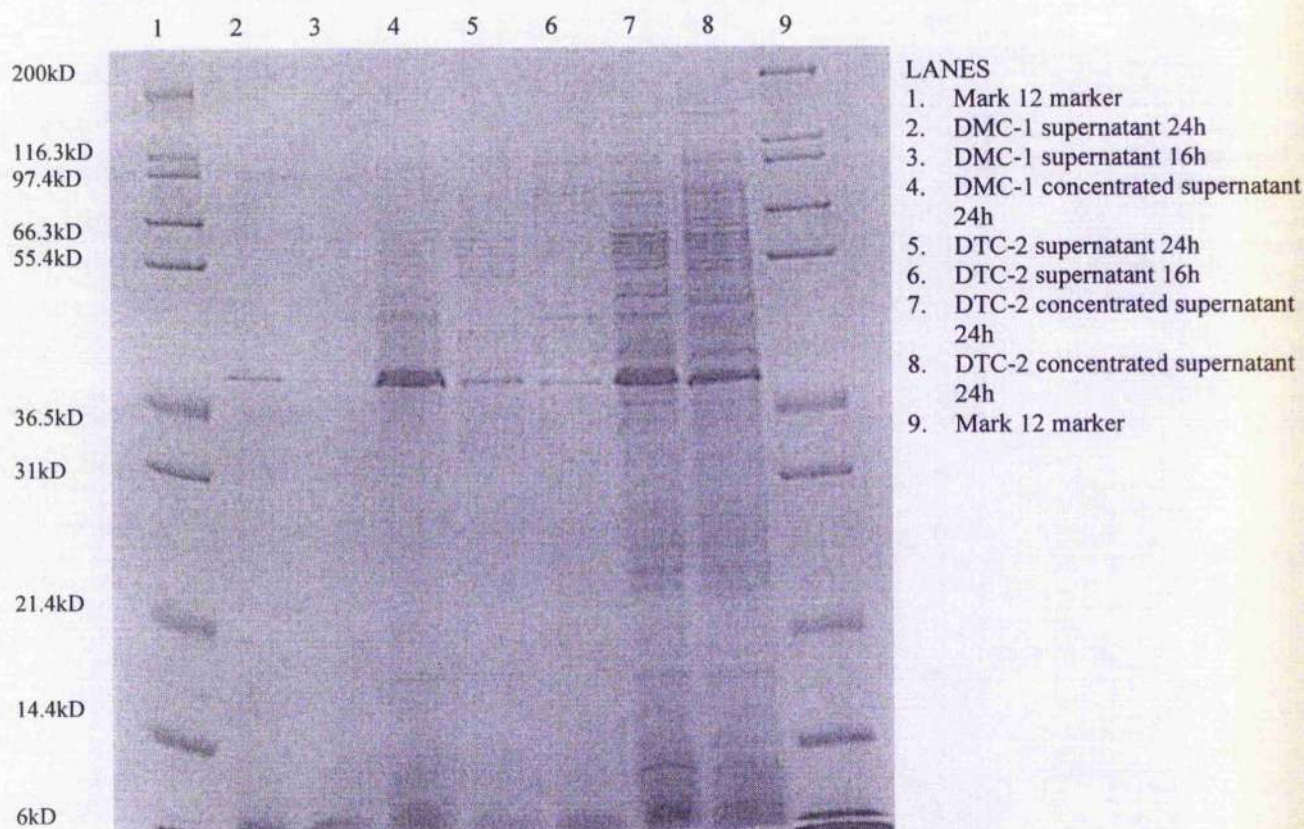


Figure 4.3. Analysis of extracellular products from virulent *V. splendidus* DMC-1 and avirulent *V. splendidus* DTC-2 culture supernatants using SDS-PAGE. Native and concentrated culture supernatants of *V. splendidus* biovar 1 strains were grown for 16 and 24 h in TSB + 1.5 % NaCl. The gel was stained with Coomassie Brilliant Blue stain. Protein standards are listed at the side in kD using Mark12 marker.

Previous results (chapter 3) showed that growth medium affected extracellular protein production and the supernatants from both MB and TSB + 1.5 % NaCl were compared in SDS-PAGE gels to assess whether differences in the production of certain proteins, e.g. the haemolysin could be detected.

The results show that the dominant band of 40 kD was only found in the culture supernatant from TSB + NaCl broth and not MB at this stage of growth (Figures 4.4 and 4.5). In Figure 4.4 only unconcentrated 24-h supernatants, before and after heat

treatment are shown. A faint band is visible in lane 3 at 36.5 kD, but not in lane 5 for the heat-treated MB culture supernatant. This analysis suggests that different proteins predominate in the culture supernatant with different growth media at this stage of growth.

When culture supernatants were concentrated by ammonium sulphate precipitation a larger range of protein bands was visible. These results supported previous evidence that there were distinct differences in proteins produced in the two growth media. Firstly, with TSB + NaCl the dominant band at 40 kD was more concentrated, but a second strong band was also visible at 65 kD. With the MB concentrated supernatant, a weak band of 40 kD was present, but the dominant band in this case was of ~36 kD, which was faintly visible in the native culture supernatant.

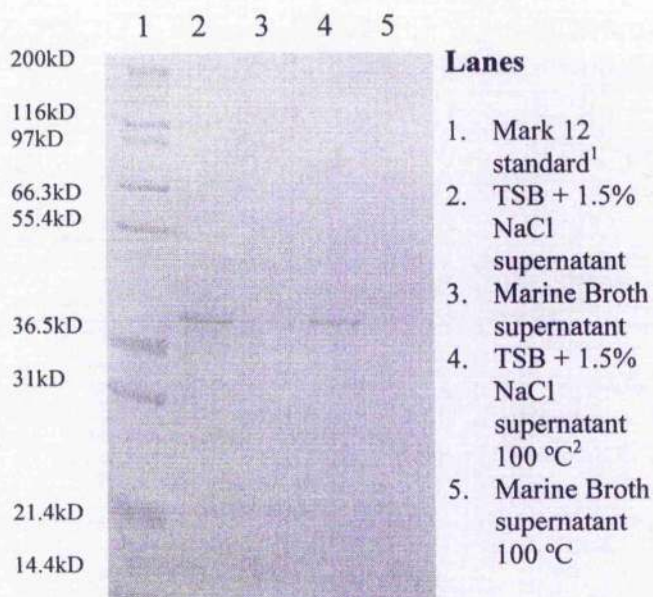


Figure 4.4. Analysis of filter-sterilised culture supernatant of 24 h cultures of *V. splendidus* DMC-1 grown in different growth media, using SDS-PAGE. *V. splendidus* DMC-1 was grown under the same conditions in two different growth media and the culture supernatant was filter-sterilised and applied to an SDS-PAGE gel. The gel was stained with Coomassie Brilliant Blue stain. ¹Mark 12 standard (Novex, Invitrogen) used as the protein standard marker. ²Filter-sterilised culture supernatant was heat-treated at 100 °C x 10 min.

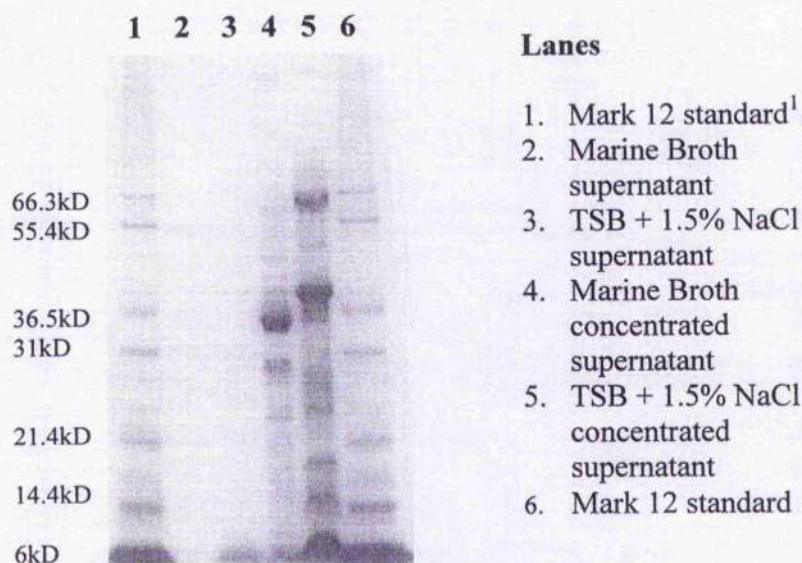


Figure 4.5. Analysis of native and concentrated filter-sterilised culture supernatant of *V. splendidus* DMC-1 grown in different growth media, using SDS-PAGE. The same material as shown in Figure 4.4 alongside concentrated material from the same growth medium. ¹Mark 12 standard (Novex, Invitrogen) used as the protein standard marker.

4.3.2. IEF

Preparative isoelectric focusing was used for separation of components of the *V. splendidus* DMC-1 culture supernatant according to their respective isoelectric point (pI) and this provided sufficient material for screening in the haemocyte assay.

Two IEF experiments were done to investigate the proteins present in the culture supernatant. In the first experiment, concentrated supernatant was separated across a pH range of 3-7. The pH gradient and distribution of protein are shown in Figure 4.6. Three protein peaks in fractions 7, 25 and 29, contained over 800 $\mu\text{g ml}^{-1}$ protein. Fractions 1-30 were tested in pools of five in the haemocyte assay at three concentrations, neat, 1/10 and 1/100 (Figure 4.7). The starting material that had been concentrated and dialysed prior to applying to the IEF gel (retentate) and SSW were used as positive and negative controls, respectively. None of the fractions had very high activity perhaps due to the processing steps of concentration, dialysis and IEF. The neat samples were all highly cytotoxic which may be due to the presence of

residual ampholines as shown by the low molecular weight material in SDS-PAGE gels of the fractions, Figure 4.9. Therefore, from the results for the 1/10 dilution, the highest toxicity was found with fractions 11 to 15, at 52.5 %. Subsequently, pairs of fractions from 11 to 20 were tested in the haemocyte assay to further localise the cytotoxic activity (Figure 4.8).

From the results obtained with 1/10 dilutions the highest cytotoxicity was found in fractions 15 + 16, although this was low (23 %). It should be noted that in this experiment the cytotoxicity for the retentate dropped from 82.1 to 55.9 for the 1/10 dilution showing possible loss of activity on storage.

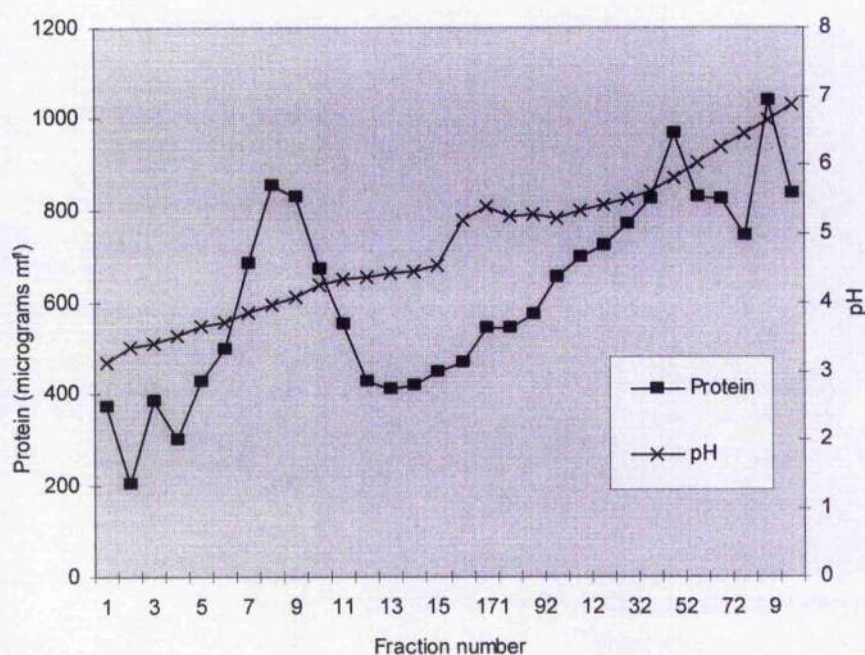


Figure 4.6. pH and protein concentration of isoelectric focusing fractions from separation of concentrated supernatant of *V. splendidus* DMC-1 (experiment 1). pH was measured with pH meter and protein was measured using a Bradford assay.

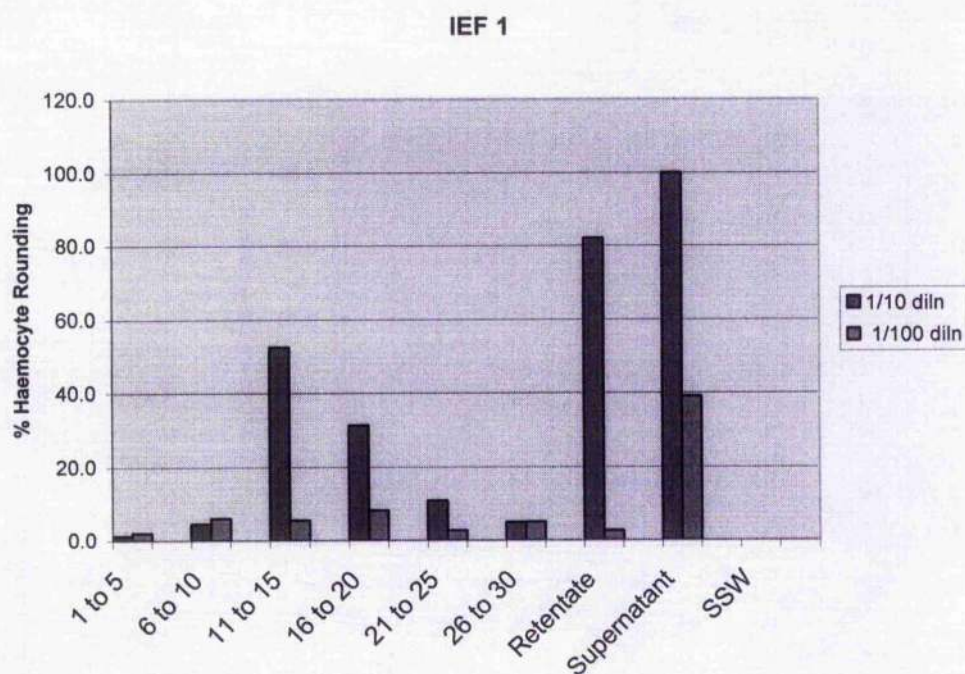


Figure 4.7. Haemocyte cytotoxicity assay of pools of 5 isoelectric focusing fractions from separation of concentrated supernatant of *V. splendidus* DMC-1 (experiment 1). Samples were applied to haemocytes at dilutions neat, 1/10 and 1/100. The retentate (starting material for the IEF after dialysis with glycine) and native culture supernatant were used as positive controls and SSW as a negative control. The neat fractions gave high cytotoxicity as a result of the presence of ampholines from the IEF gel and results have been omitted from the graph.

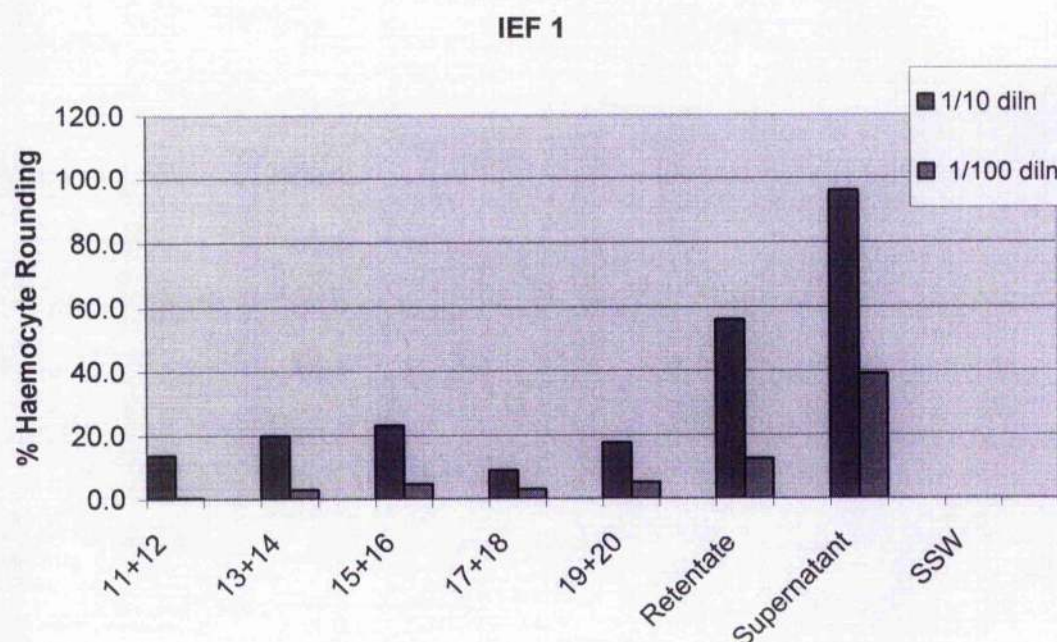


Figure 4.8. Haemocyte cytotoxicity assay of pooled groups of 2 fractions from isoelectric focusing of concentrated supernatant of *V. splendidus* DMC-1 (experiment 1). (see Figure 4.7 for description).

The fractions were also applied to SDS-PAGE gels to analyse the proteins present in each fraction. No protein bands were visualised in fractions 1-10 (gel not shown), and Figure 4.9 shows the results for fractions 11-30. The dominant protein band in the fractions was a 40 kD protein. However, other bands were present. The darkening at the bottom of the gels is a result of traces of ampholines eluted from the IEF gel.

Overall, the recovery in fractions 1-30 was about 40 % with Figure 4.7 showing a distinct peak in the profile of the activity in fractions 11 to 15. However, this is lost in Figure 4.8, with only a slight peak in activity in fractions 15 to 16. It was estimated that up to a third of the input may have been recovered.

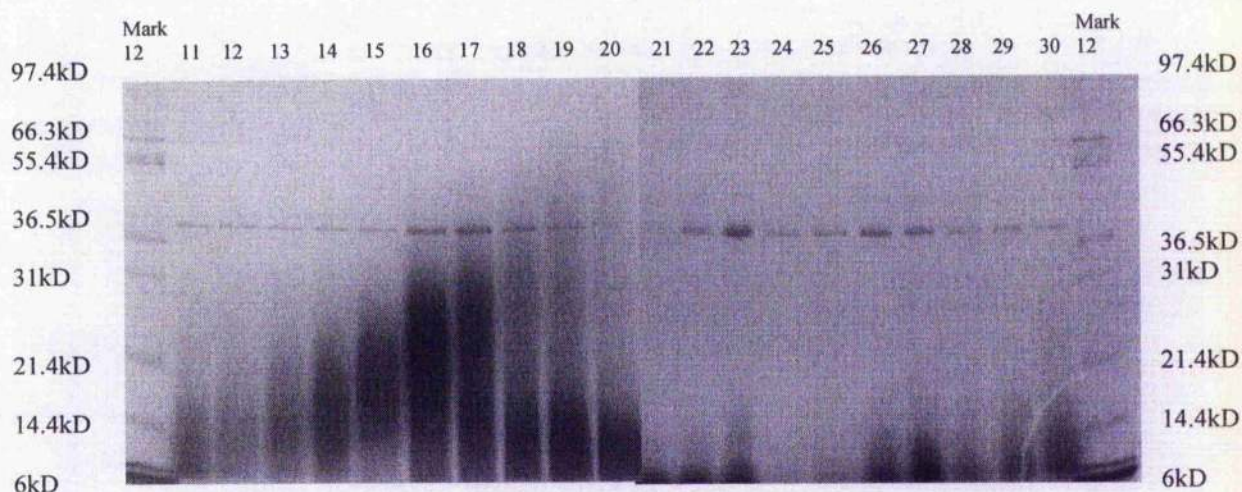


Figure 4.9. SDS-PAGE analysis of isoelectric focusing fractions 11-30. The dominant band in all fractions was of 40 kD. Mark 12 protein standard marker was used, protein sizes shown at side of gel in kD. The gel was stained with Coomassie Brilliant Blue stain.

It was considered possible that the 40 kD protein band observed in SDS-PAGE might be associated with cytotoxic activity as a peak of intensity occurred in fractions 16-17 on SDS-PAGE, coinciding with increased cytotoxic activity (Figure 4.7). However, the group of fractions with the highest cytotoxic activity were fractions 11-15.

Fraction 13 was selected for study by separation on SDS-PAGE and electro-transfer of the proteins to PVDF membrane for N-terminal sequence determination. The 40 kD protein had an isoelectric point of 4.43 and the N-terminal sequence is shown in Figure 4.10. A comparison was made with database sequences using the FASTA program on the European Bioinformatics Institute website and the sequence was found to have 95 % homology with the OmpU precursor of *V. vulnificus*, 89.5 % with *V. parahaemolyticus*, and 53 % homology with *V. cholerae* OmpU precursor. Also shown is *E. coli* Omp C which had 53 % homology.

<i>V. splendidus</i> DMC-1 Omp	AELYNQ DGTHLDMGGRAEA
<i>V. vulnificus</i> OmpU	AELYNQ DGTSLDMGGRAEA
<i>V. parahaemolyticus</i> OmpU	AELYNQ DGTSLEMGGRAEA
<i>V. cholerae</i> O1 OmpU	STV Y SAK G TS LE VGGRAEA
<i>E. coli</i> Omp C	AEVYN KDGNK LD LYGKVTA

Figure 4.10. Comparison of the N-terminal sequence of *Vibrio splendidus* DMC-1 40 kD protein and other bacterial outer membrane N-terminal porin sequences. Letters in bold text represent the amino acids with homology to the Omp of *V. splendidus*.

A second IEF experiment was carried out in exactly the same way as the first IEF experiment and in this case the pH ranged from 3-9 (Figure 4.11). The distribution of protein differed from that in Figure 4.6, with peaks of protein in fractions 12, 13, 15 and 22 greater than $800 \mu\text{g ml}^{-1}$. It appeared that a better separation of proteins was obtained in experiment 2 with more defined peaks of protein compared to experiment 1.

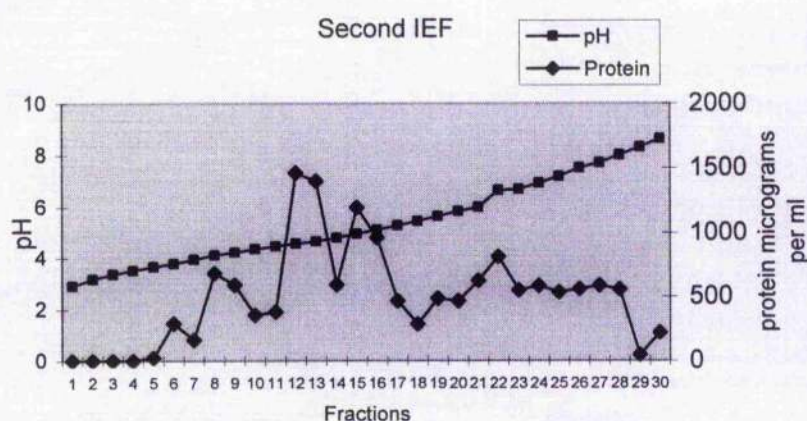


Figure 4.11. pH and protein concentration of isoelectric focusing fractions from separation of concentrated supernatant of *V. splendidus* DMC-1 (experiment 2). pH was measured with pH meter and protein was measured using a Bradford assay.

The fractions were tested in the haemocyte assay (Figure 4.12) and the high cytotoxicity in fractions 1-5 probably arose from the low pH as no protein was detected in these fractions (Figure 4.11 and 4.13). Overall, little cytotoxicity was found in fractions from this experiment and the intensity of stained protein bands on SDS-PAGE was lower than in the previous experiment (Figure 4.13).

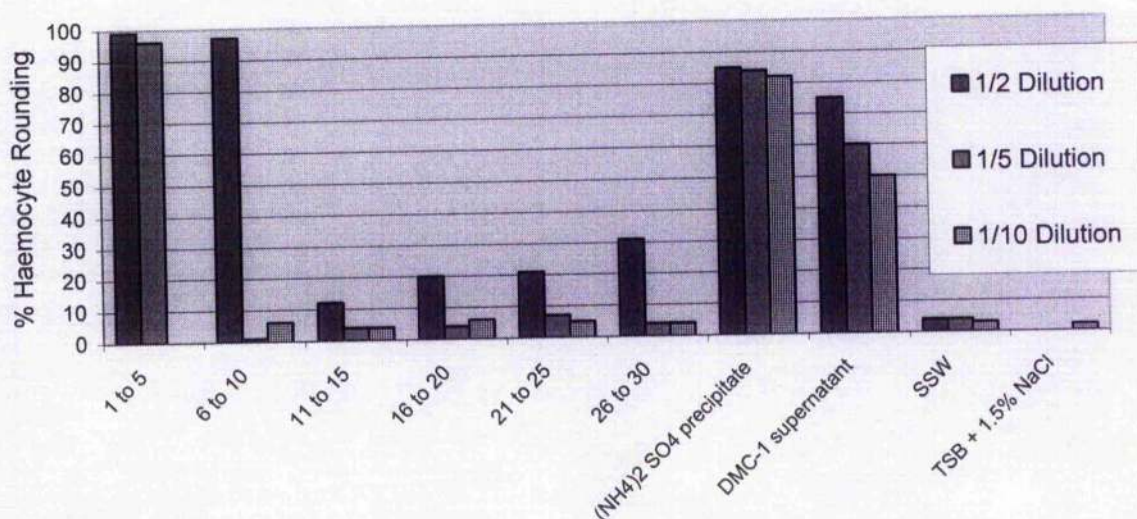


Figure 4.12. Haemocyte cytotoxicity assay of pooled groups of 5 fractions from isoelectric focusing (experiment 2). Samples were applied to assay at neat, 1/2, 1/5 and 1/10 dilutions. The positive controls were concentrated culture supernatant (ammonium sulphate precipitated) and native culture supernatant, with the negative controls of SSW and growth medium. The neat results were omitted as in Figures 4.7 and 4.8.

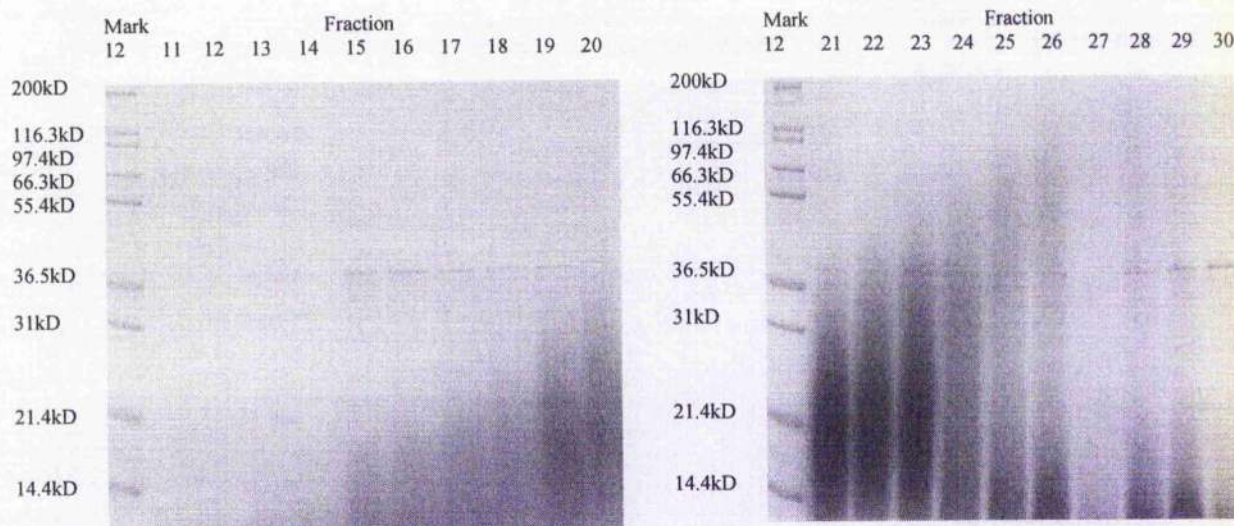


Figure 4.13. SDS-PAGE analysis of isoelectric focusing fractions 11-30 (experiment 2). The dominant band in all fractions was at 40 kD. The Mark 12 protein standard marker was used (sizes in kD), the gel was stained with Coomassie Brilliant Blue stain.

As previously found, no protein bands were detected in fractions 1-10, and fractions 11-30 again contained a dominant band of 40 kD. The other bands present were of ~30 and 35 kD.

4.3.3. Protease assays

Zymogram gels were used to identify the presence of proteases within the bacterial supernatant with these yielding clear bands where the protease(s) has digested the substrate against a dark blue background. Culture supernatants from three *V. splendidus* biovar 1 strains were tested, including DMC-1. The results show clearly that strain DTC-5 has strong protease activity. DMC-1 showed slight protease activity, although this was not clearly visible in the photograph (Figure 4.14).

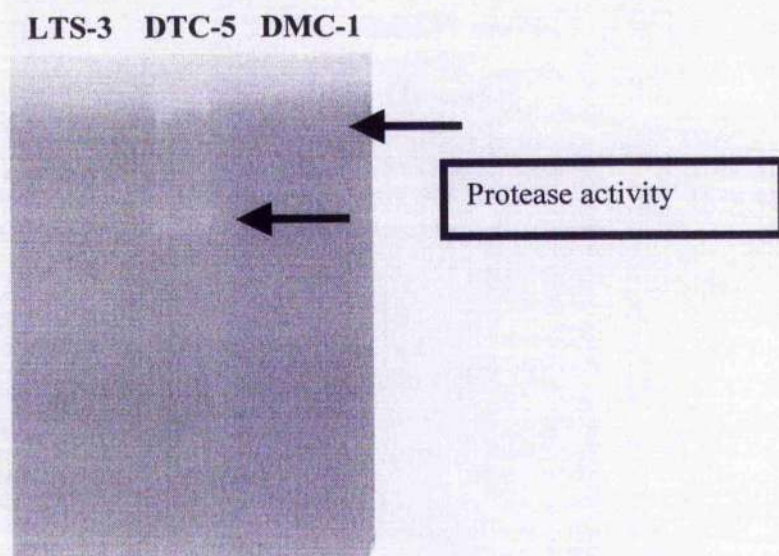


Figure 4.14. Zymogram protease gel with filter-sterilised culture supernatant of *V. splendidus* DMC-1, DTC-5 and LTS-3. Zones of clearing are indicated by the arrows (protease activity).

From these results, further assays were carried out using the protease substrates, elastin-Congo red, azoalbumin and azocasein. Only strain DMC-1 was used in these assays. Protease activity detected was $240 \mu\text{g ml}^{-1}$ trypsin equivalent with elastin-Congo red as substrate, $49 \mu\text{g ml}^{-1}$ of trypsin equivalent against azoalbumin and $10 \mu\text{g ml}^{-1}$ of trypsin equivalent against azocasein. *V. splendidus* DMC-1 was also found to be positive for phospholipase and caseinase production.

4.3.4. Haemolysin

As was described in chapter 3, *V. splendidus* DMC-1 produces a broad-range haemolysin, the production of which depends on the growth medium used (Table 3.2). Filter-sterilised 24 h MB culture supernatant of *V. splendidus* DMC-1 had a haemolysin titre of 1/256 (Table 4.1), and haemolytic activity was abolished by heating at 100°C for 10 min (Figure 3.8). From this it is shown that the haemolysin is heat sensitive.

A range of *V. splendidus* biovar 1 strains, both sucrose-positive and -negative, were tested in previous turbot larval trials to distinguish virulent and avirulent strains. This was compared with haemolysin production (Table 4.1) and overall, cultures of the virulent strains had higher haemolysin titres than the avirulent strains. This could be as a result of more than one haemolysin being produced by *V. splendidus* biovar 1

strains, or the haemolysin may not be expressed to the same extent in the avirulent strains.

Strain	Turbot larvae trial ¹	Haemolysin titre ²
<i>V. splendidus</i> DMC-1	Virulent	1/256
<i>V. splendidus</i> DTC-5	Virulent	1/256
<i>V. splendidus</i> HNF-8	Virulent	1/256
<i>V. splendidus</i> LTS-3	Virulent	1/256
<i>V. splendidus</i> DTC-2	Avirulent	1/128
<i>V. splendidus</i> DTY-1	Avirulent	1/32
<i>V. splendidus</i> LTH-3	Avirulent	1/2
<i>V. splendidus</i> LTS-4	Avirulent	1/128

Table 4.1. Larval toxicity and haemolysin titres for numerous *V. splendidus* strains.

¹data from Thomson (2001).

²This study.

Further work was concentrated on characterisation of the haemolysin (Chapter 5) to determine if it was an important virulence factor of *V. splendidus* DMC-1.

4.4. Discussion

V. splendidus DMC-1 was chosen as the main isolate for study due to its high virulence towards turbot larvae in a previous study (Thomson, 2001). In an attempt to understand the pathogenic mechanisms involved in virulence, virulent and avirulent isolates were compared in selected experiments.

As high cytotoxic activity was detected in the culture supernatant of this *V. splendidus* strain the nature of extracellular products was investigated to determine whether they are likely to contribute to virulence. The overall results from this chapter have identified three potential virulence factors in the culture supernatant. These are all extracellular proteins released from the bacterial cell, a 40 kD outer membrane protein (OMP), protease(s) and a broad-range haemolysin(s) or cytotoxin(s). Another possible virulence factor identified was a species-specific adhesin.

4.4.1. OMP

The dominant protein band identified in culture supernatant of TSB +1.5 % NaCl was a 40 kD protein and the most cytotoxic fraction from isoelectric focusing of culture

supernatant corresponded with one of the peaks of occurrence of this protein band. The protein at 40 kD was present throughout all fractions which may be as a result of the protein associating with bacterial LPS that could have prevented the protein from being separated completely into fractions during isoelectric focusing. The N-terminal sequence of this protein identified it as being very closely related to OmpU of both *V. vulnificus* and *V. parahaemolyticus*. This 40 kD band was also present in the concentrated supernatant of the MB (Figure 4.5), however in this medium a second protein of 36 kD was dominant. In preliminary studies with the two types of growth medium it was shown that catabolite repression down-regulated haemolysin production (Appendix 10) and this might also regulate the switching of these proposed OMPs.

In the genus *Vibrio*, OMPs function primarily as porins for the entry of hydrophilic and low molecular weight molecules including, iron, phosphate and sugars that may then be transported across the inner membrane (Lang and Palva, 1993, McCarter and Silverman, 1987, Tashima *et al.*, 1996). They can also be involved in the attachment to inanimate and animate surfaces (Sperandio *et al.*, 1996). Bacterial fimbriae and outer membrane proteins (OMPs) are bacterial components that mediate attachment to specific tissues by acting as adhesins (Sperandio, *et al.*, 1995; Chakrabarti *et al.*, 1996). For example OmpU of *V. fischeri* plays a role in the initiation of colonisation of the light organ of juvenile squid (Aeckersberg *et al.*, 2001).

Intestinal colonisation by bacteria is complex involving sequential or simultaneous expression of several factors. OmpU of *V. cholerae* is the dominant OMP of this species and OmpU expression is co-regulated with expression of critical virulence factors such as cholera toxin (CT) and toxin co-regulated pilus (TCP), suggesting that OmpU is an important virulence factor (Sperandio *et al.*, 1995). It has been proposed that in *V. cholerae* OmpU is a colonisation factor involved in adherence (Sengupta *et al.*, 1992; Sperandio *et al.*, 1995). OmpU has also been shown to have haemagglutinating activity (Chakrabarti *et al.*, 1996). However, Nakasone and Iwanaga (1998) did not consider OmpU of *V. cholerae* to be involved in adherence *in vivo*.

In *V. cholerae*, ToxR is the transcriptional activator that is responsible for coordinating the expression of the outer membrane porins, OmpU (38 kD) and OmpT (40 kD). OmpU is induced when OmpT is repressed (Provenzano *et al.*, 2000; Okuda *et al.*, 2001), which is critical for bile resistance (Wibbenmeyer *et al.*, 2002, Karunasagar *et al.*, 2003), virulence factor expression, and intestinal colonisation (Provenzano and Klose, 2000; Provenzano *et al.*, 2001.) Therefore, this suggests a relationship between porin production and pathogenesis mechanisms (Provenzano and Klose, 2000; Provenzano *et al.*, 2001) and might also explain the difference in protein expression in the two types of growth medium.

Overall, the OMPs produced by *V. splendidus* may play an important role in the pathogenesis of this organism in the same manner as those discussed above.

4.4.2. Protease

The results from the use of zymogram gels and protease assays with azo-substrates and milk-agar show that there is slight protease activity in the supernatant of *V. splendidus* DMC-1. There was also a strong band of protease activity detected for strain DTC-5, also a *V. splendidus* biovar 1 isolate. Zhang and Austin (2000) found that in a range of *V. harveyi* isolates the expression of proteolytic activity was variable.

Protease activity is common in the vibrios with many of the proteases produced being metalloproteases possessing a zinc atom, and being immunologically cross-reactive to each other (Norqvist *et al.*, 1990; Häse and Finkelstein, 1993). The proteases act not only to process and activate protein toxins but also directly as toxic factors causing oedematous or haemorrhagic skin lesions or disturbance of host defense system (Farrell and Crosa, 1991).

The proteases secreted by bacteria are well known for their roles as virulence determinants (Häse and Finkelstein, 1993), and multiple proteases have been implicated as virulence factors in *V. harveyi* (Zhang and Austin, 2000), *V. cholerae* (Booth *et al.*, 1984), *V. anguillarum* (Norqvist *et al.*, 1990), *V. vulnificus* (Kothary and Kreger, 1987) and also *Pseudomonas aeruginosa* (Caballero *et al.*, 2001).

For *V. splendidus*, Gatesoupe *et al.* (1999) suggested that peptidase enzymes were important in virulent strains of this organism as arylaminopeptidase was only detected in virulent strains. These aminopeptidases are also detected in oral bacteria where they are thought to play a role in tissue destruction in periodontal disease (Suido *et al.*, 1986). However, as mentioned in chapter 1, production of this arylaminopeptidase was not detected in the *V. splendidus* DMC-1 isolate. Due to the low protease activity detected for the *V. splendidus* DMC-1 supernatant, no detailed study was carried out to further characterise the protease(s).

4.4.3. Adhesin

Haemagglutination of bacteria is closely related to the adhesiveness of the organism to the surface of cells and is believed to play an important role in the pathogenicity of the bacteria. Preliminary studies were carried out to investigate haemagglutinating activity, which was only seen with turbot erythrocytes suggesting that a turbot-specific adhesin is produced by this *V. splendidus* DMC-1 isolate. Such an adhesin may be involved in adherence of bacteria to the gut of the turbot larvae. Adhesins can be fimbrial or afimbrial, the latter including OMPs, LPS and extracellular polysaccharides. Therefore, as already discussed, the OmpU-like protein identified by N-terminal sequencing, may be an adhesin of this strain, particularly if its expression is regulated in the same manner as other virulence factors.

In *V. cholerae*, Hanne and Finkelstein (1982) identified and characterised different haemagglutinins that played a role in adherence, and Sasmal *et al.* (1995) showed *V. cholerae* 0139 strains to be highly adhesive with haemagglutinating properties. Toranzo *et al.* (1983) found that *V. anguillarum* isolated from striped seabass produced strong haemagglutinins for fish erythrocytes, whereas non-pathogenic vibrios did not produce haemagglutinins. However, Trust *et al.* (1981) found no correlation between strain virulence and haemagglutination of fish erythrocytes with *V. anguillarum* strains. Nevertheless, it is well established that adhesion is an essential prerequisite of many bacteria for colonisation and eventual disease.

Therefore, the production of an adhesin by *V. splendidus*, together with other virulence factors may contribute to the pathogenicity of this organism. Further study is required on the adhesive properties of this isolate. This would involve adhesion

assays using tissue culture cell lines of turbot origin to measure levels of bacteria that adhere as this would give an indication of bacterial adhesiveness to a relevant substrate. The invasive capabilities of the isolate could also be measured in a similar manner. Further assays could involve the addition of specific inhibitors, such as sugars to the adhesion assay, to determine if they inhibited adhesion by blocking attachment sites (Wang and Leung, 2000).

4.4.4. Haemolysin

Vibrio splendidus was identified as being a haemolytic isolate from turbot larvae feeding on *Artemia* brine shrimps at about day 20 post-hatch (Thomson, 2001). Investigation of the haemolysin was regarded as important because of the association between poor larval rearing success and the presence of high concentrations of haemolytic bacteria in the cultures. Haemolysin is also the most widely distributed toxin in pathogenic vibrios and plays various roles in the infection process.

The culture supernatants for TSB + 1.5 % NaCl contained little haemolytic activity (titre $\leq 1/8$), with the haemolytic activity being much greater in the MB cultures (titre 1/256). The factors required for expression of high levels of haemolysin activity have not been investigated further but preliminary studies showed that addition of glucose to cultures inhibited the production of haemolysin (Appendix 10). As this is not a component of MB it may explain why there is better production of haemolysin with this medium. Further work in this area needs to be carried out.

The crude haemolysin in the supernatant of *V. splendidus* showed various biological activities. It was capable of lysing red blood cells from various sources, and was cytotoxic to cultured cells such as TV1-S4 and CHSE-214 (results not shown). The *V. splendidus* haemolysin was heat-labile (100 °C for 10 min), as is the HlyA of *V. cholerae* (Iwanaga and Ichinose, 1991) and *V. mimicus* haemolysin, (Shinoda *et al.*, 1993).

The comparison of haemolysin expression between four virulent and four avirulent isolates of *V. splendidus* showed that the virulent isolates expressed higher haemolytic activity, (Table 4.1). Therefore, this may suggest that *V. splendidus* haemolysin plays

an important role in the pathogenicity of this organism in combination with the other virulence factors discussed. Further work was carried out to characterise the *V. splendidus* haemolysin, this work is discussed in chapter 5.

Chapter 5. Characterisation of the haemolysin of *Vibrio splendidus*

5.1. Introduction

As was discussed in chapter 4, the production of haemolysin in pathogenic *Vibrio* species is common. In fact haemolysins produced by several different species of vibrio have been found to be similar but not identical, (Iida and Honda, 1997). A haemolysin gene has been identified from the fish pathogen *V. anguillarum* that has homology with El Tor haemolysin of *V. cholerae*, *Aeromonas hydrophila* haemolysin and *A. salmonicida* haemolysin (Hirono *et al.*, 1996). The importance in pathogenicity of the *V. anguillarum* haemolysin is unknown. The similarities between these haemolysins in important pathogens provides good reason for further study of the *V. splendidus* haemolysin.

Further study of the *V. splendidus* haemolysin as a major virulence factor required further characterisation of the toxin and the mechanism of action was investigated to assess how the toxin caused damage to cells. Purification was attempted followed by molecular characterisation to identify the *V. splendidus* haemolysin and enable comparisons with other haemolysins. The main aim of the work in this chapter was to understand how this toxin plays a role in the pathogenesis of the strain.

5.2. Materials and Methods

5.2.1. Haemolysin assay

This was carried out as described in section 2.2.8 and 3.2.2.

5.2.2. Effect of temperature on binding of haemolysin to erythrocytes

To determine whether haemolysin could bind to and lyse erythrocytes at low temperature a 1 ml suspension of 2, 5, 10 and 20 % (v/v) washed sheep erythrocytes was added to 1 ml of crude culture supernatant and incubated on ice for 1 h. The suspensions were centrifuged at 4,000 x g for 4 min at 0 °C, and the supernatant removed and held on ice. The pellet of blood cells was resuspended in PBS and incubated at 20 °C for 1 h. The supernatant was titrated using the haemolysin assay.

5.2.3. Concentration of Haemolysin

5.2.3.1. Dialysis

Dialysis was performed on concentrated supernatant to remove excess salt from the solutions for equilibration with the buffer used for column chromatography or for IEF, or to concentrate supernatant by reducing the volume in the dialysis tubing. The tube was sealed and immersed in buffer (for dialysis) or immersed in solid flakes of polyethylene glycol 6000 to concentrate the sample by removal of water.

5.2.3.2. Ammonium sulphate precipitation

This was carried out in accordance with section 4.2.1.

5.2.3.3. Amicon Filtration

Diaflo ultrafiltration membranes (Amicon) PM 10 (>10,000 MW), PM 30 (> 30,000 MW), XM 50 (50,000 MW) YM 100 (>100,000 MW) and XM 100 (>100,000 MW) were also used for concentration of haemolysin solutions. All membranes are pre-treated with glycerin to prevent drying and the YM series are pre-treated with sodium azide. Membranes were removed from their protective envelope using forceps and these preservatives were removed by soaking the filters briefly in distilled water followed by flushing with distilled water prior to use. The YM series of filters were pre-soaked in distilled water for 1-2 h with periodic water exchanges, before use. All membranes were placed with the skin (glossy) side up in a 50 ml Amicon ultrafiltration chamber. The unit was sealed and held on ice with a stirrer underneath to mix the solution in the chamber. Sample was added to the unit via a pipette and the unit pressurised to 40 psi. Once the volume had been reduced to 5 ml the pressure was released and the retentate removed. The filtrate was also retained and both were tested for haemolytic activity in the haemolysin assay.

5.2.3.4. Tangential flow concentration

Eight litres of MB cultures of *V. splendidus* DMC-1 were incubated for 16 h at 20 °C and the culture supernatant collected after centrifugation at 9,000 x g for 10 min. A Filtron Omega miniset unit with a 10 kD Omega filter was used to concentrate the culture supernatant using a peristaltic pump to circulate the supernatant through the closed system. The system was initially flushed with 0.1 M NaOH to remove the

preservative, sodium azide/glycerin or any other protein, from the membrane. After flushing the system with distilled water, culture supernatant, held on ice in a 10 L flask was pumped through the system to concentrate proteins of *V. splendidus* DMC-1. The concentrate was tested for haemolytic activity using the haemolysin assay, before use in further purification experiments.

5.2.4. Osmotic protection studies on haemolysin-treated erythrocytes

Several known haemolysins lyse cells by formation of transmembrane pores but this colloid-osmotic lysis can be inhibited if the suspending solution contains a high concentration of molecules unable to pass through the pore. A range of molecules of known viscometric radius can then be used to estimate the diameter of the transmembrane pore. A 2.5 ml volume of a 0.4 % erythrocyte suspension was dispensed into 12 test tubes (13 mm x 100 mm) and equal volumes of 30 mM polyethylene glycol (PEG) solutions (Table 5.1) were added in duplicate to the tubes containing the erythrocyte suspensions, keeping one duplicate set aside to act as a positive haemolytic control. The tubes were incubated at room temperature for 10 min to allow equilibration of solutions before addition of 100 μ l of concentrated supernatant (haemolysin titre of 1/1024) diluted to give 100 HU ml⁻¹, to one set of the duplicate tubes and 100 μ l of PBS was added to the second set of tubes to act as negative controls. The tubes were vortexed gently and A600 nm measured using a Corning Colorimeter 252. Readings were taken every two min for 20 min and then every 10 min for 1 h for the tubes containing crude haemolysin (concentrated supernatant). The control tubes were measured every 10 min.

Polyethylene glycol (PEG)	Viscometric radius ¹
PEG 300	0.48 nm
PEG 400	0.56 nm
PEG 600	0.69 nm
PEG 1000	0.89 nm
PEG 1500	1.1 nm

Table 5.1. Polyethylene glycol (PEG) viscometric radius sizes. ¹from Kuga (1981)

5.2.4.1. Effects of metal ions and other agents on haemolytic activity

Solutions of 10 mM inorganic salts and 100 mM solutions of EDTA and dithiothreitol (DTT) were prepared in PBS and 100 µl of these solutions added to 0.9 ml of concentrated *V. splendidus* DMC-1 supernatant to give final concentrations of 1 mM and 10 mM, respectively. The solutions were incubated for 1 h at room temperature and the haemolytic activity measured. Ten-fold dilutions of the metal ion solutions and inhibitors were used as negative controls and concentrated supernatant was used as a positive control.

Further work with the zinc sulphate and manganese sulphate solutions was carried out using a range of concentrations, 0.1 mM, 1 mM, 5 mM and 10 mM. Equal volumes (2.5 ml) of the metal ion solutions and 0.4 % suspension of washed sheep erythrocytes were mixed in 12 mm test tubes and 100 µl of toxin added and the solution was vortexed. The kinetics of haemolysis was measured over a selected time period using a Corning 252 colorimeter to measure absorbance at 600 nm. Tubes containing metal ions with PBS added were used as negative controls and concentrated supernatant alone was used as a positive control.

5.2.5. Column chromatography

5.2.5.1. Ion-exchange chromatography

A HiTrap selection kit (Pharmacia) of small ready-packed columns of ion-exchange material was used to optimise concentration of the toxic factor. Both cation (sulphopropyl, SPFF and carboxymethyl, CMFF) and anion (quaternary ammonium, QFF, and DEAE) exchangers were tested. Columns were initially washed with 1 M NaOH to strip any material from columns, and then rinsed thoroughly with 20 mM phosphate buffer. Concentrated *V. splendidus* DMC-1 supernatant was dialysed against the same buffer for 2 h at 4 °C prior to loading onto the columns. Volumes of 5 ml of dialysed sample were applied using syringes and the columns were then eluted with 5 ml of phosphate buffer. Fractions were collected in 0.5 ml volumes and tested for haemolytic activity and activity was titrated using the haemolysin assay.

5.2.5.2. Q-column

The HiTrap selection kit suggested that the ideal ion-exchange material was quaternary ammonium (Q-column). Therefore, a preparative column (25 cm x 2 cm)

was prepared by packing Q-Sephadex into a column and allowing it to settle. Using a peristaltic pump the column was washed with 100 ml of 20 mM Bis-Tris to equilibrate the Q-Sephadex. Concentrated DMC-1 culture supernatant was dialysed against 20 mM Bis-Tris buffer for 2 h at 4 °C and the dialysed sample loaded onto the column at a flow rate of 2 ml min⁻¹. The column was washed with 20 mM Bis-Tris and 0.4 M NaCl was applied after detection of haemolysin. Fractions of 2 ml were collected at 4 °C and the fractions were tested for protein and haemolytic activity. The column was finally washed with 20 mM Bis-Tris and cleaned with 0.1 M NaOH.

5.2.5.3. Molecular weight chromatography

A 70 cm x 2 cm column was packed with 100ml of S-200 Sephacryl and was equilibrated by washing with 200 ml of 20 mM Bis-Tris, 0.15 M NaCl + bovine serum albumin (0.1 mg ml⁻¹), pH 6.8. The haemolytic fractions obtained from the Q-column were pooled and dialysed against 20 mM Bis-Tris for 1 h at 4 °C prior to application to the molecular weight column. Sample and buffer was loaded onto the column at a rate of 24 ml h⁻¹ and 3 ml fractions collected and tested for haemolytic activity; protein concentration was estimated from initial samples (no BSA added) from absorbance readings at 280 nm, assuming that a 1 mg ml⁻¹ solution of protein gave A_{280 nm} = 1. The column was eluted with >100 ml of 20 mM Bis-Tris containing 0.15 M NaCl. BSA was included in later experiments to act as a carrier protein, no protein was measured from these fractions.

5.2.5.4. Hydrophobic interaction chromatography

A 15 cm x 1.5 cm column was packed with phenyl-Sepharose CL 4B (Pharmacia) and equilibrated with 10 bed volumes of starting buffer (0.05M Tris-HCl, pH 7.0, with (NH₄)₂SO₄). The concentration of (NH₄)₂SO₄ used in the buffer was optimised to enable elution of the active material from the column and is stated in the text for each experiment. Pooled fractions from the ion-exchange column (Q-Sepharose) were concentrated in an Amicon filtration unit using a PM10 filter and dialysed against the starting buffer for 1 h prior to loading onto the phenyl-Sepharose column. Haemolytic activity was tested prior to loading, and 1 ml sample was loaded onto the column at a flow rate of 0.2 ml min⁻¹. The column was eluted using 10 ml of starting buffer. Fractions were eluted using a suitable concentration of (NH₄)₂SO₄ at a flow rate of 0.4 ml min⁻¹. Fractions of 2 ml were collected and tested for haemolytic activity; those that were positive were titrated using the haemolysin assay. The absorbance at 280 nm was also measured to estimate the protein concentration.

5.2.5.5. Concentration of column chromatography fractions.

This was done as previously described using an Amicon filtration unit with PM10 filters (section 5.2.3.3) or by dialysis against solid polyethylene glycol 6000 (section 5.2.3.1). Active fraction were pooled and placed in 6 mm diameter dialysis tubing of molecular weight cut-off of 12-14 kD. The dialysis tubing was overlaid with flakes of PEG 6000 and held on ice for 1 h. This method reduced the volume of the fractions by approximately 50 % to concentrate proteins in the active fractions.

5.2.6. Native polyacrylamide gel electrophoresis

Tris-glycine gels (4-20 %) (Novex, Invitrogen) were used. The sample was prepared by adding one volume of sample buffer (2X concentration) to one volume of sample. Tris-glycine running buffer (1X) was used in the upper and lower chambers of the Novex gel system apparatus. A volume of 10 μ l of sample was applied directly onto the gel, in duplicate on either side of the gel. The gel was run at 50 V for 3 h with a cooling system to keep the apparatus and gel at 4 °C. The gel was sliced into two sections with the duplicate samples on each gel. One half was fixed and stained as for SDS-PAGE gels (section 4.2.2.), the other half was overlaid with 1 % agarose containing 5 % sheep blood. The latter gel was held at room temperature and monitored for haemolytic zones.

5.2.7. Silver staining

The native PAGE gel was fixed in 30 % methanol, 10 % acetic acid solution in distilled water for 10 min. The fixative was removed and replaced with 10 % glutaraldehyde solution for 30 min followed by three washes of 2 min in distilled water to remove glutaraldehyde, and storage in distilled water overnight. The gel was then washed for 15 min in distilled water prior to soaking in 5 μ g ml⁻¹ dithiothreitol (DTT) for 30 min. The DTT solution was removed and, without rinsing, 0.1 % silver nitrate solution was added for 30 min. The gel was rinsed twice in distilled water then twice in developer solution (37 % formaldehyde (formalin) in 3 % sodium carbonate) before incubation in developer solution until protein bands developed to the required intensity. A 5 ml volume of 2.3 M citric acid was added and mixed for 10 min to neutralise the developer. The gel was finally washed in distilled water.

5.2.8. Isoelectric Focusing (IEF)

See section 4.2.3.

5.2.9. Molecular studies of the haemolysin gene

5.2.9.1. Genomic library construction

5.2.9.1.1 Extraction of DNA using BIORAD kit

Bacterial cultures were grown overnight with shaking at 20 °C in 5 ml volumes of MB in plastic universal bottles and 250 µl of the cultures were placed in 1.5 ml microfuge tubes and held on ice. The cultures were centrifuged at 15,000 x g for 1 min to pellet cells, the supernatant carefully removed and 300 µl of Genomic DNA Lysis solution was added to resuspend the pellet. The samples were incubated at 80 °C for 5 min to lyse the cells and 1.5 µl of RNase A solution (4 mg ml⁻¹) was added to the cell lysate. The sample was mixed by inverting the tube and incubated at 37 °C for 45 min before cooling to room temperature and addition of 100 µl of Protein Precipitation buffer. Samples were vortexed vigorously for 20 sec and then centrifuged at 15,000 g for 3 min. The precipitated proteins formed a compact pellet and the supernatant (containing the DNA) was poured into microfuge tubes containing 300 µl 100 % isopropanol. Samples were mixed gently by inverting the tubes before centrifugation at 15,000 x g for 1 min to pellet the DNA. The supernatant was discarded and tubes were briefly inverted on clean absorbent paper. A 300 µl volume of 70 % ethanol was added, tubes were inverted several times to wash the DNA, and centrifuged at 15,000 x g for 1 min. The ethanol was carefully poured off the DNA pellet and tubes were inverted again onto clean absorbent paper and allowed to air-dry for 15 min. DNA Hydration Solution, 50 µl, was added to hydrate the DNA and the sample was incubated at 65 °C for 1 h to accelerate hydration. Finally, the sample was vortexed to mix the contents and centrifuged briefly to collect the sample at the bottom of the tube. DNA extracts were stored at -20 °C for further use.

5.2.10. Plasmid genomic library construction

5.2.10.1. Chromosomal DNA preparation

Conditions were established for partial digestion of chromosomal DNA using varying concentrations of the restriction enzyme *Sau3A* (Promega) to obtain DNA fragments

ranging between 2-4 kb. The digested DNA was extracted from an agarose gel or used directly from the restriction digest.

5.2.10.2. DNA purification from restriction digest

The DNA was extracted twice with equal volumes of phenol:chloroform (24:1, Sigma). Two volumes of ice-cold ethanol were added and the mixture was held on ice for 5 min before centrifugation at 15,000 x g for 10 min to pellet DNA. The DNA was resuspended in 100 µl Tris-HCl pH 7.0 and stored on ice for further use or at -20 °C for long term storage.

5.2.10.3. Plasmid DNA preparation

The plasmid vector pUC18 was amplified after transformation into chemically-competent *E. coli* TOP10 cells and was purified using a QIAprep plasmid preparation kit (Qiagen) (Appendix 8). The plasmid was digested using the restriction enzyme *Bam*HI (Promega) followed by purification as described above (5.2.10.2).

5.2.10.4. Dephosphorylation of Plasmid DNA

After purification, the plasmid DNA was dephosphorylated by addition of 10 µl calf intestinal phosphatase (CIP) buffer and 0.34 µl of CIP (Promega). The samples were incubated at 37°C for 30 min before addition of EDTA and SDS to final concentrations of 5 mM and 5 %, respectively. Samples were mixed, 100 µl 10 mg ml⁻¹ of Proteinase K solution added, incubated at 56 °C for 30 min and cooled to room temperature. Phenol:chloroform extraction was carried out twice and 0.1 vol. of 3 M sodium acetate and 2 vol of ethanol were added to precipitate DNA. Samples were incubated on ice for 15 min, centrifuged and pellets washed with 70 % ethanol. The final DNA pellet was resuspended in 10 µl TE buffer, pH 7.8. The concentration of DNA was assessed visually on agarose gels using DNA electrophoresis.

5.2.10.5. Ligation of pUC18 and DMC-1 chromosomal DNA

A 100 ng quantity of pUC18 vector was transferred into a sterile microfuge tube, and an equimolar concentration of DMC-1 purified DNA fragments was added; 7.5 µl distilled water was added and the mixture heated to 45 °C for 5 min to melt any cohesive termini that had reannealed. Samples were transferred to ice. For the

ligation reaction, 1 μ l of 10X T4 DNA ligase buffer, 0.8 μ l of T4 ligase (Promega) and 1 μ l of 5 mM ATP were mixed with the following:

- A. 100 ng of dephosphorylated pUC18
- B. 100 ng of dephosphorylated pUC18 + 100 ng of DMC-1 DNA fragments
- C. 100 ng of pUC18 not dephosphorylated

Samples were incubated at 15 °C for 1 h or overnight at 4 °C.

5.2.10.6. Transformation of ligated DNA

Aliquots of *E. coli* TOP10 cells were thawed on ice from -80 °C storage and 1 μ l volumes of the ligation reactions A-C were added to the cells and held on ice for 5 min. The reaction mixtures containing the cells were heat-shocked at 42 °C for 30 sec, then returned to ice and 250 μ l of SOC medium was added to each sample after which they were incubated at 37 °C for 1 h. Selective plates containing X-Gal, IPTG, 150 μ g ml⁻¹ ampicillin and 5 % sheep blood were used to culture 130 μ l of transformation mixtures in duplicate for each sample. Plates were incubated at 37 °C overnight, and the transformants counted and analysed.

5.2.11. Lambda (λ) library

5.2.11.1. DNA fragments

To create a λ library it was necessary to obtain large fragments of genomic DNA in the range of 4-12 kb for ligation into the λ ZAP vector. A restriction enzyme digestion series with varying concentrations of the restriction enzyme *Sau*3A was carried out against preparations of *V. splendidus* DMC-1 genomic DNA to obtain the correct size of fragments at optimum enzyme concentration. The DNA fragments were separated on 0.7 % agarose gels in TE buffer. The fragments were extracted from the agarose gel using a Qiagen Gel Purification kit and the DNA fragments analysed for concentration and size using gel electrophoresis.

5.2.11.2. ZAP Ligation

The DNA fragments were used for ligation into λ ZAP express library. The ligation reaction and a control reaction was set up as follows:

- 1.0 μ l ZAP express vector (1 μ g ml⁻¹)
- 1.6 μ l pBub test insert (0.4 μ g)/2.5 μ l of DMC-1 DNA (0.31 μ g)

0.5 μ l 10x ligase buffer

0.5 μ l 10 mM ATP

dH₂O up to 5 μ l

2 units of T4 ligase (0.6 μ l of 3 units μ l⁻¹ stock)

The ligation mixtures were incubated at 4 °C overnight.

The host strain *E. coli* XL1-Blue MRF['] was inoculated into 5 ml of LB broth supplemented with 0.2 % maltose and 10 mM MgSO₄ for transformation of the ligated DNA. The culture was incubated at 37 °C with shaking for 4-6 h until a culture density of OD₆₀₀ = 1 was reached.

5.2.11.3. ZAP vector packaging reaction

The packaging extracts were removed from -80 °C and partially thawed on ice. ZAP/DNA ligations were added immediately to the packaging extracts, the samples were mixed and tubes were pulsed in a microcentrifuge to collect material at the bottom of the tube. The samples were incubated at room temperature for 2 h and 500 μ l SM buffer and 20 μ l chloroform were added. The tubes were centrifuged briefly to sediment any debris and the supernatant containing the phage was used for titration. This phage titration was carried out using the bacterial host *E. coli* XL1-Blue MRF['] which was cultured to mid-exponential phase and adjusted to OD₆₀₀ = 0.5 using 10 mM MgSO₄ solution. The supernatant containing the phage was mixed with the bacteria in two ways:

1 μ l of packaged reaction + 200 μ l of *E. coli* XL1-Blue MRF['] cells

1 μ l of 1/10 dilution of packaged reaction + 200 μ l of *E. coli* XL1-Blue MRF['] cells

The phage preparations were incubated with bacteria at 37 °C for 15 min to allow attachment to the bacterial cells. NZY top agarose was melted and allowed to cool to 48 °C and 15 μ l of 0.1 M IPTG, 50 μ l of X-Gal (40 mg ml⁻¹) and 2-3 ml of NZY top agarose was added to phage/bacterial mixture and the mixture poured immediately onto the surface of the NZY agar plates and the agarose allowed to set before incubation at 37 °C overnight. The plaques were visible within 6-8 h, but colour change required overnight incubation. The white plaques were formed by phage containing genomic DNA inserts. Both blue and white plaques were counted to

determine the concentration of the phage library in plaque forming units ml^{-1} (pfu ml^{-1}).

5.2.11.4. Amplification of λ ZAP Library

The phage library was amplified using 220 mm square agar plates with NZY agar. The same protocol was followed for amplification of the phage as was carried out for the titration, with the volume of bacterial cells increased from 200 μl to 600 μl per plate. The phage sample volume was also increased from 1 μl to 4 μl and was added to 10-20 ml NZY top agarose. After growth and formation of the plaques, the plates were overlaid with 8-10 ml SM buffer and incubated overnight at 4 °C with gentle agitation to allow phage to diffuse into the SM buffer. The bacteriophage suspension was recovered from each plate with an additional rinse with 2 ml of SM buffer. The suspensions were pooled into sterile tubes, chloroform added to a final concentration of 5 % (v/v) and suspensions were incubated at room temperature for 15 min to kill any contaminating bacteria. Debris was removed by centrifugation at 500 x g for 10 min, the supernatant recovered and transferred to fresh tubes. If the solution was cloudy then the chloroform stage was repeated followed by centrifugation. Finally, chloroform was added to 0.3% (v/v) and the amplified phage stock was mixed with DMSO (7 % final concentration), and 1 ml aliquots were stored at -80 °C. The titre of amplified phage was measured and recorded.

5.2.11.5. Single-clone excision from the ZAP library

Escherichia coli XL0LR and XL1-Blue MRF' were inoculated into 50 ml of LB broth in baffled 250 ml Erlenmeyer flasks. The cultures were incubated overnight at 37 °C. Six white plaques were cored from an agar plate from titration experiments with a 10^{-2} dilution of phage stock. These were transferred into sterile microfuge tubes containing 500 μl of SM buffer and 20 μl of chloroform. Samples were vortexed to release phage and were incubated at room temperature for 1-2 h or 4 °C overnight.

The bacterial cultures were harvested by centrifugation at 1,000 x g for 10 min and were resuspended in 10 mM MgSO_4 solution to $\text{OD}_{600}=1$. A 200 μl volume of *E. coli* XL1-Blue MRF' cells was mixed with 250 μl of phage and 1 μl of ExAssist

helper phage. The mixture was incubated at 37 °C for 15 min, 3 ml of NZY broth was added and the sample was incubated at 37 °C for 2.5 to 3 h. The tubes were heated at 65 °C for 20 min, centrifuged at 1,000 x g for 15 min and the supernatants, containing the pBK-CMV phagemid vector packaged as filamentous phage, were decanted into sterile tubes.

From the *E. coli* XL0LR cell suspension, 200 µl was added to twelve tubes, which then received either 100 µl or 10 µl of each of the six phage preparations. The samples were incubated at 37 °C for 15 min, 300 µl of NZY media added and the samples further incubated at 37 °C for 45 min. A 200 µl volume of each cell/phage sample was spread in duplicate onto LB agar plates containing 50 µg ml⁻¹ kanamycin, and the plates incubated at 37 °C overnight. Colonies were enumerated and a selection were chosen for further study. Each colony selected contained the pBK-CMV double stranded phagemid with the cloned DNA insert.

The colonies were isolated and inoculated into LB broth containing 50 µg ml⁻¹ of kanamycin and grown at 37 °C overnight. The cultures were treated for plasmid isolation using the QIAprep kit (Qiagen) as previously described (section 5.2.10.3 and Appendix 8). Restriction digests using *Eco*RI were carried out on the plasmid preparations to assess the insert size.

5.2.12. Screening of libraries

5.2.12.1. Plasmid library

Selected transformants that contained an insert of DMC-1 genomic DNA were inoculated into LB broth containing 150 µg ml⁻¹ of ampicillin. Cultures were grown overnight at 37 °C and were processed using a QIAprep plasmid preparation kit (Appendix 8) to isolate pUC18 + DMC-1 DNA insert. The plasmid preparations were digested with *Eco*RI and the resulting digests were visualised on a 0.7 % agarose gel after electrophoresis. This initial screening was carried out to assess the average DNA insert size, to ensure that 2 to 4 kb or larger fragments were inserted into the vector.

The transformants were screened on selective blood agar plates to analyse the genomic plasmid library for haemolytic clones, and these were selected and analysed. The plasmids from these clones were isolated as before, analysed to determine the DNA insert size, and the plasmids sent for sequencing with M13 forward and reverse primers to DBS Genomics to identify the potential haemolysin gene(s) within the genomic DNA insert.

Fragments containing the plasmid vector and truncated DNA insert were religated using the above ligation protocol (section 5.2.10.5) and smaller fragments were ligated into new pUC18 vectors. The new plasmid constructs were transformed into *E. coli* TOP10 cells as before and were assessed for haemolytic activity on selective blood agar plates. The plasmids were isolated from *E. coli* TOP10 cells as before, restriction digested with suitable enzymes and the insert size assessed on 0.7 % agarose gel using DNA gel electrophoresis. These restriction digest results enabled a restriction map of the original insert to be plotted and the resulting plasmid constructs were further sequenced with M13 forward and reverse primers at DBS Genomics.

5.2.12.2. Lambda library

An overnight culture of *E. coli* XL1-Blue MRF' was grown and used to screen the λ ZAP DMC-1 library. The culture was harvested and resuspended to an optical density of 0.5 at 600 nm, 200 μ l of the bacterial suspension was added to 20 bijou bottles, and 5 μ l of phage suspension, diluted to 10^{-4} in SM buffer was added to each bijou bottle. The samples were incubated at 37 °C for 30 min and 3 ml of top NZY agarose plates containing 100 mM IPTG and 40 mg ml^{-1} X-Gal, was added to each bijou bottle. The mixture was poured onto the surface of NZY agar plates containing 5 % blood. Plates were incubated at 37 °C overnight and analysed for any haemolytic plaques. Those that were found to be haemolytic were cored using a Pasteur pipette into 500 μ l of SM buffer containing 20 μ l of chloroform. Mixtures were vortexed briefly and left to stand at room temperature for 1-2 h to extract phages. The material was titrated as previously described to confirm the haemolytic activity associated with the phages. The original phage library was repeatedly screened to identify further haemolytic plaques, and suspensions of the original and amplified stocks of λ ZAP DMC-1 phage were stored in small volumes in 7 % DMSO at -80 °C.

5.2.13. Transposon Mutagenesis

5.2.13.1. Tn5

Initial work was carried out using Tn5 (transposon) located within the vector pUT::miniTn5CmlacZ, created by Hosie (1998). The host cells, *E. coli* Sm10/ λ pir were used and the plasmid was introduced into these cells using electroporation.

5.2.13.2. Preparation of electrocompetent cells

A 5 ml culture of *E. coli* Sm10/ λ pir was grown overnight in LB broth at 37 °C. The culture was washed twice in PBS, the optical density measured and the volume adjusted to obtain OD₆₀₀ of 1 ($= 1 \times 10^9$ cfu ml⁻¹). A volume of 1 ml of this culture was inoculated into 1 L of 2YT broth, prewarmed to 37 °C, and the culture grown at 37 °C, in a shaking incubator. Growth rate was monitored by measuring OD₆₀₀ periodically. Once the culture reached exponential phase the cells were chilled on ice for 30 min and the culture was centrifuged at 4,000 x g for 20 min at 4 °C. The bacterial pellet was resuspended in 1 L of ice-cold 1 mM Hepes buffer (pH7.0), centrifuged for a further 10 min and washed with 500 ml of 1 mM Hepes buffer. The pellet was resuspended in 20 ml of 1 mM Hepes buffer containing 10 % glycerol. A further centrifugation step was carried out at 3,000 x g for 20 min with final resuspension of pellet in 3 ml of 10 % glycerol. The cells were stored at -80 °C in microfuge tubes in 200 μ l volumes for latter use in electroporation.

5.2.13.3. Electroporation of *E. coli* Sm10/ λ pir

A volume of 50 μ l of electrocompetent cells was placed into an electroporation cuvette held on ice and 1-2 μ l of plasmid vector, pUT::miniTn5CmlacZ was added to the cuvette. Cells were subjected to electroporation using a Biorad Gene Pulser at 2.5 V, resistance 200 Ohms. SOC medium (1 ml) was added to the cuvette immediately after application of the electrical current and the electroporated cells were incubated with shaking at 37 °C for 1 h. Five 200 μ l volumes were plated out onto selective LB plates containing 50 μ g ml⁻¹ chloramphenicol and 150 μ g ml⁻¹ ampicillin to select for cells containing the plasmid vector. Inoculated plates were incubated at 37 °C overnight and the bacterial colonies containing the plasmid vector were selected and used as donor strains in transposon mutagenesis experiments.

5.2.13.4. Mutagenesis with Tn5

The donor cells containing Tn5 were sensitive to streptomycin, the vector contained an ampicillin resistance marker and the transposon carried a chloramphenicol resistance marker. As the recipient *V. splendidus* cells were sensitive to chloramphenicol and resistant to streptomycin this enabled selection of recipient strains containing the plasmid/transposon. The host strain was grown in 50 ml of LB broth and the target strain was grown in 50 ml of MB, both in 250 ml baffled flasks at 37 °C and 20 °C, respectively. Both strains were grown overnight and the two strains mixed in ratios of 1:2, 1:3 and 1:4, and made up to 3 ml with SSW. Filter-mating was carried out using filter-units containing 0.2 µm polycarbonate filters. The mixtures of bacteria were passed through sterile filter units in 3 ml volumes, the filters removed from the units and placed aseptically onto LB15 + MgSO₄ agar plates. The plates were incubated at 20 °C for 8 h to allow conjugative transfer of plasmid from the donor to the recipient strain. After incubation, the filters were removed, washed with 3 ml of SSW and 200 µl was plated out onto both MA and MA + 5 % sheep blood with 50 µg ml⁻¹ of chloramphenicol and 200 µg ml⁻¹ of streptomycin. Plates were incubated at 20 °C until growth of any mutants was seen.

Spontaneous resistance of *E. coli* Sm10/λpir to streptomycin and *V. splendidus* to chloramphenicol was tested by plating out 100 µl of each culture onto selective LB and MA plates containing 200 µg ml⁻¹ of streptomycin and 50 µg ml⁻¹ of chloramphenicol, respectively. Plates were incubated for up to a week and monitored for growth.

An alternative mutagenesis experiment used Tn5 from a different source in the vector pUTminiTn5Cmlux, obtained from Dr M. Lynch, Division of Infection and Immunity, University of Glasgow. The vector was purified using a QIAprep plasmid preparation kit and was electroporated into *E. coli* Sm10/λpir cells. The host strain *E. coli* Sm10/λpir was grown in 5 ml LB containing 30 µg ml⁻¹ chloramphenicol and 150 µg ml⁻¹ ampicillin at 37 °C. The recipient strain, *V. splendidus* DMC-1 was grown in 5 ml of MB at 20 °C. The optical density of cultures was measured at 600 nm after growth overnight to estimate the cell numbers in each culture. This enabled known ratios of donor and recipient cells to be set up. The appropriate volumes for the

required ratios were centrifuged at $15,000 \times g$ to pellet bacterial cells, the supernatants were removed and the pellets resuspended in 1 ml of fresh growth medium. The cultures were further centrifuged and pellets resuspended in 100 μ l of growth medium. Volumes of 50 μ l of each culture were mixed together and $10 \times 10 \mu$ l volumes were spotted onto LB/MA plates along with controls for each strain. The plates were incubated overnight at 20 °C and the mating spots were removed from the plate surface using a sterile nichrome loop and inoculated into separate microfuge tubes containing 100 μ l of fresh LB medium. A 100 μ l volume of ffm phage was added to samples and incubated for 30 min at room temperature before spreading 200 μ l of each mixture onto TCBS plates containing 30 μ g ml⁻¹ chloramphenicol. Plates were incubated at 20 °C overnight.

5.2.13.5. Ffm Phage preparation

An overnight culture of *E. coli* Sm10/ λ pir grown in 5 ml of LB broth was diluted 100-fold in fresh LB broth and grown for 2 h at 37 °C. A volume of 50 μ l of phage suspension was added to this culture which was further incubated at 37 °C with agitation for 2.5-3 h. The culture optical density was monitored and cell lysis assessed throughout. A 'stringy' consistency of the culture indicated the occurrence of cell lysis and at this stage a few drops of chloroform were added to the culture to stop lysis, and prevent resistant *E. coli* cells from further growth. The chloroform was allowed to settle and the upper, aqueous layer containing the phage particles was transferred to fresh tubes. The phage particles were stored at 4 °C for up to 4 months. The efficiency of phage against *E. coli* was tested by using a lawn plate of *E. coli* with spots of diluted phage placed on the surface. After incubation overnight clear zones appeared in areas where phage had lysed *E. coli* cells effectively.

5.2.13.6. Phage experiments against *V. splendidus* DMC-1

Before using the phage for lysis of *E. coli* donor cells, the recipient strain *V. splendidus* was tested to confirm that it was resistant to the phage. A 100 μ l volume of overnight *V. splendidus* DMC-1 culture was spread onto MA plates and dilutions of phage were spot inoculated onto the plate surface. Plates were incubated overnight at 20 °C and inspected for any signs of lytic effects.

5.2.13.7. Mutagenesis with Tn10

The transposon Tn10 located in the plasmid vector pBSL181 was kindly supplied by Dr J.B. Andersen, BioCentrum DTU, Denmark in the host cells *E. coli* S17- λ pir. The host strain was grown overnight at 37 °C in LB broth, and the plasmid was purified using a QIAprep kit. The plasmid was electroporated into *E. coli* Sm10/ λ pir as before, to provide a more suitable host strain for use in mutagenesis based on antibiotic selection.

5.2.13.8. Optimisation experiments

A series of 5 ml volumes of culture medium, either LB with varying concentrations of NaCl (1 %, 1.5 %, 2 % and 2.5 %) or MB were used. The temperature for growing strains was optimised using 20 °C, 30 °C and 37 °C. The growth conditions were also varied by using shaking or static conditions. These temperature and media changes were also applied at the mating stage of mutagenesis. The method of conjugation was changed in accordance with Solano *et al.* (2000) instead of using filter mating, different ratios of donor:recipient were tested. In addition to this, different buffers were used for resuspension of conjugated strains. Finally, the concentrations of antibiotics used in selective plates were altered. Different combinations of the above were used in mutagenesis experiments in an attempt to optimise conditions and produce a high number of transposon mutants.

5.2.13.9. Protocol applied for Tn10 mutagenesis

The donor strain *E. coli* Sm10/ λ pir was grown in LB broth at 37 °C and the *V. splendidus* DMC-1 was grown in MB at 20 °C, both cultures (50 ml) were agitated in baffled 250 ml flasks overnight. The optical density of each culture was measured to estimate the cell numbers for each strain and enable a ratio of 1:1 to be made. Viable cell counts were taken of each culture to determine the actual ratio established. The donor strain was spotted onto the surface of LB15 + MgSO₄ agar plates in 20 μ l volumes and dried in a laminar flow hood. The recipient strain was spotted on to the top of the donor strain in the same volume and the plates dried again. Several ratios were set up based on the cell number estimation so as to obtain an actual 1:1 ratio. The mating plates were incubated at 20 °C overnight, colonies on the viable count

plates were counted and total cell number for each strain were recorded. Only plates from the ratios that achieved an actual viable count ratio of 1:1 were processed further. The mating spots were scraped from the plate surface into microfuge tubes containing 200 μ l of NSS + MgSO₄. The spots were resuspended and plated onto selective MA plates containing 5% sheep blood before incubation at 20 °C overnight for mutants to grow.

5.2.13.10. Screening of mutants

5.2.13.10.1. Identification tests

Mutants were selected and inoculated into 96-well plates containing 150 μ l of MB with 30 μ g ml⁻¹ chloramphenicol and were grown overnight at 20 °C with agitation. Glycerol was added to final concentration of 5 % and plates were stored at -80 °C for further use. Simple biochemical tests were used to initially assess whether the mutants were true *V. splendidus*. Non-haemolytic mutants were inoculated onto TCBS, spot inoculated onto filter paper containing 1 % solution of N, N, N, 'N'-tetramethyl-p-phenylenediamine dihydrochloride to test for oxidase activity and Gram stained to check staining and morphology. The haemolysin-negative mutants were tested in comparative studies with DMC-1 to assess whether any other differences had occurred due to the transposon insertion. The strains were grown in 50 ml of MB in 250 ml baffled flasks and their growth rate measured over a period of 48 h with regular optical density measurements at 600 nm. The bacterial supernatants from these cultures were filter-sterilised and studied using the haemolysin assay, Bradford assay and API-ZYM assay, using protocols as described before.

5.2.13.10.2. Genomic DNA Purification-Wizard, Promega

Genomic DNA was isolated from transposon mutants using the Wizard Genomic DNA Purification Kit from Promega. A 1 ml volume of an overnight culture was centrifuged at 15,000 x g for 2 min and supernatant was discarded. The cells were lysed with 600 μ l of Nucleic Lysis Solution and mixtures were pipetted gently to ensure mixing. Samples were incubated at 80 °C for 5 min, cooled to room temperature and 3 μ l of RNase solution was added and mixed. The samples were incubated at 37 °C for 60 min, cooled to room temperature and 200 μ l of Protein Precipitation solution was added and solutions vortexed to precipitate protein. The samples were incubated on ice for 5 min, centrifuged at 15,000 x g for 3 min and the

supernatant transferred into clean microfuge tubes containing 600 µl of isopropanol to precipitate the DNA. The samples were mixed gently and centrifuged at 15,000 x g for 2 min. The DNA pellet was washed with 70 % ethanol, the ethanol was then aspirated and the pellet air-dried for 15 min before rehydration in 100 µl of Rehydration Solution for 1 h at 65 °C or overnight at 4 °C.

5.2.13.10.3. DNA purification from restriction digests

See section 5.2.10.2

5.2.13.10.4. Determination of transposon insertion site by PCR

Primers were designed against the known sequence of the chloramphenicol Tn10 resistance marker of the cat cassette using the program Primer 3 (Figure 5.1).

```
AGCTTTCCTGACGGAATGTTAATTCTCGTTGACCCCTGAGCACTGATGAATCCCTAATGATTTTGGTAAAAATCATTAAAGTTAAGG
TGGATACACATCTTTGTCATATGATCCCGGATCGGTGGAGCTCGAATTGACGGTCCGAATTTCTGCCAATCATCCGCTTATTATCA
CTTATTCAGGCGTAGCAACCCAGGCGTTTAAGGGCACCATAAATGACCTTAAATAAATTACGCCCGCCCTGCCACTCATCGCAGTA
CTGTTGTAATTCAATTAAGCATTCGCCGACATGGAAGCCATCACAAACGGCCATGATGAACCTGAATCGCCAGCGGUCATCAGCACCT
TGTCGGCTTGCGTATAATAATTGUCCATGGTGAAAACGGGGCGAAGAAGTTGTCCATATTGGCCACGTTTAAATCAAACTGGTG
AAACTCACCAGGGATTGGCTGAGACGAAAACATATTCTCAATAAACCCCTTAGGGAAATAGGCCAGGTTTTCACCGTAAACCGC
CACATCTTCCGAATATATGTGTAGAAACTGCCGAAATCGTCGTGGTATTCACCTCCAGAGCGATGAAAACGTTTCAGTTTGCTCAT
GGAAAACGGTGAACAGGGTGAACACTATCCATATACGAGCTCACCGTCTTTCATTGGCCATACGAAATCCGGATGAGCATTCAT
CAGGCGGGCAAGAAATGTCATATAAGGCCGGATAAAACTTGTGCTTATTTTCTTTACGGTCTTTAAAAAGGCCGTAATATCCAGCT
GAACCGCTCTGGTTATAGGTACATTGAGCAACTGACTGAATGCTCAGAAATGTTCTTTACGATGCCATGGGATATATCAACGGTG
GTATATCCACATGATTTTCTCCATTTTAGCTTCCCTAGCTCCTGAAAATCTCGATAACTCAAAAAATACGCCCGGTAGTGATCT
TATTTCAATTATGGTCAAAGTTGGAACTCTTAGCTGCCGATCAAGCTCTCATTTTCGCCAAAATTTGGCCAGGGCTTCCCGGTAT
CAACAGGACACCAAGGATTATTTATTTCTGCGAAGTGATCTTCCGTACAGGTATTTATTCGGACGCGTCTCGGTACCGGGCCGC
CCCTCGAGGTGACGGTATCGATAAGCTTGATATCGAAT
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Figure 5.1. Mini-Tn10 Cloning vector sequence from DMC-1-M2. Primers highlighted in red were used to amplify the chloramphenicol cassette located within the transposon Tn10 in PCR of the potential mutants.

The PCR reactions were set up using chromosomal DNA preparations from a selection of transposon mutants using the Wizard DNA extraction kit (Promega). Both positive *E. coli* Sm10/ λ pir(pBSL181miniTn10Cm) and negative *V. splendidus* DMC-1 templates were included in the PCR reaction. The solution required for 12 reactions was as follows:

dH₂O 214.5 µl

10 X Buffer (100 mM Tris-HCl, pH 9.0, 15 mM MgCl₂; 500 mM KCl; 0.1 % gelatin

(w/v); 1 % (v/v) Triton X-100 (Qiagen))

	30.0 μ l
dNTPs	6.0 μ l
Taq Polymerase	1.44 μ l
Primers:	
cat forward	12.0 μ l
cat reverse	12.0 μ l

The PCR master mix was dispersed in 23 μ l volumes and 2 μ l of template DNA was added. The PCR conditions were 1 cycle at 95 °C for 15 min, 35 cycles at 94 °C, 50 °C, 72 °C each for 1 min, followed by 1 cycle at 72 °C for 10 min. The PCR products were visualised on 0.7 % agarose gel using DNA electrophoresis.

Restriction enzyme sites were identified within both the plasmid vector and the transposon to select an enzyme that would digest the vector whilst keeping the transposon intact (Figures 5.2 and 5.3). The best restriction enzyme for digestion of the plasmid vector pUC18 and genomic DNA was *Sph*I. The pUC18 vector was digested as follows:

dH ₂ O	15.3 μ l
10X Buffer	2.0 μ l
BSA	0.2 μ l
<i>Sph</i> I enzyme	0.5 μ l
Plasmid DNA	2.0 μ l

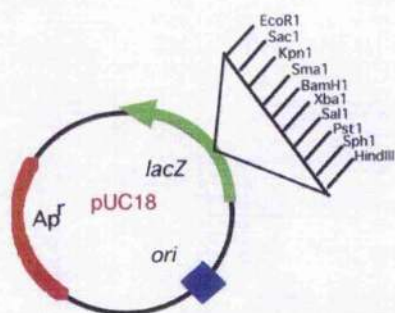


Figure 5.2. pUC18 vector. The polycloning site has been expanded to highlight the restriction enzymes sites. Ampicillin resistance marker on plasmid is shown in red (Ap^r).

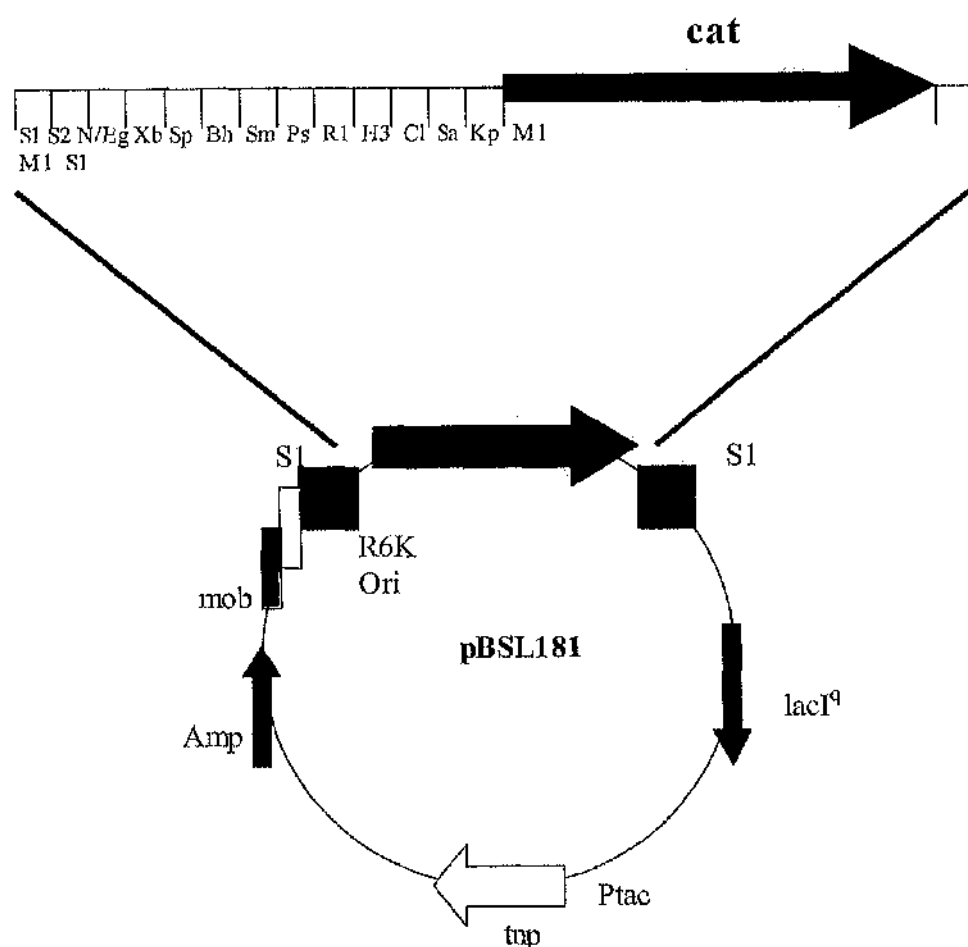


Figure 5.3. pBSL181 (6.0 kb) with Tn10 with the polycloning site expanded to highlight the restriction enzyme sites and the chloramphenicol resistance cassette (cat). The filled boxes indicate the 110-bp inverted repeats consisting of the outermost 70-bp of IS10 right embedded in 40 bp of the λ *cl* gene sequence terminating at a *Hind*III site as in pNK2884 (Kleckner *et al.*, 1991). The *tnp* gene encodes Tn10 transposase. Bb, *Bam*HI; Cl, *Cla*I; Eg, *Eag*I; H3, *Hind*III; Kp, *Kpn*I; M1, *Mlu*I; N, *Not*I; Ps, *Pst*I; R1, *Eco*RI; Sa, *Sal*I; S1, *Sac*I; S2, *Sac*II; Sm, *Sma*I; Sp, *Spe*I; Xb, *Xba*I; Amp, ampicillin resistance marker. (modified from Alexeyev and Shokolenko, 1995).

After incubation at 37 °C for 1 h the digested plasmid DNA was purified and dephosphorylated. The chromosomal DNA of the haemolysin-negative mutant was digested with a dilution series of *SphI* enzyme to find the optimum enzyme concentration that produced fragments of a wide size range. The enzyme was used at two-fold serial dilutions that ranged from 10 to 0.313 Units.

The genomic DNA digest with *SphI* was set up as follows:

dH ₂ O	See Table 5.2
10X buffer	2 µl
<i>SphI</i> enzyme	See Table 5.2
Chromosomal DNA	5 µl

Amount of <i>SphI</i> (Units)	Volume of Enzyme solution (µl)	Volume of dH ₂ O (µl)
10	1.0	12
5	0.5	12.5
2.5	0.25	12.75
1.25	0.125	12.88
0.625	0.06	12.94
0.313	0.03	12.97

Table 5.2. Volume of *SphI* restriction enzyme for the restriction digest of haemolysin-negative mutant chromosomal DNA to obtain a wide size-range of fragments.

The chromosomal DNA digestions were incubated at 37 °C for 1 h, then held on ice and 3 µl of 0.2 M EDTA was added to stop the digestion reaction. The preparations were analysed by electrophoresis in a 0.7 % agarose gel. The *SphI* digestion was repeated on chromosomal DNA with the optimal enzyme concentration and with DNA concentration increased by 4-fold.

5.2.13.10.5. DNA purification of plasmid and chromosomal DNA and dephosphorylation of plasmid vector DNA.

See section 5.2.10.

5.2.13.10.6. Ligation of plasmid vector with digested chromosomal DNA

See section 5.2.10.5. For DMC-1-M2 and M3 the ligations were set up as follows:

1. pUC18 4 μ l + DNA fragments 4 μ l
2. pUC18 1 μ l + DNA fragments 2 μ l

5.2.13.10.7. Transformation of pUC18/M2 or M3

See section 5.2.10.6.

5.2.13.10.8. Digestion of plasmids containing M2/M3 insert

The plasmid preparations were digested with the restriction enzyme *Sph*I to identify the fragment insert size. A master mix was made up for 9 digests:

dH ₂ O	146.7 μ l
10X Buffer	18.0 μ l
BSA	1.8 μ l
<i>Sph</i> I enzyme	4.5 μ l

The master mix was divided into 19 μ l aliquots and 1 μ l of plasmid DNA was added for each digest. The digestion reactions were incubated at 37 °C for 1 h and DNA fragments visualised following electrophoresis on a 0.7 % agarose gel. The plasmids were sent to the Durham Sequencing Service at the DBS Genomics, University of Durham for sequencing with M13 forward and reverse primers.

Further sequencing was carried out using primers designed from sequence data returned from DBS genomics (see Appendix 2-3). The sequences of interest were entered into a primer design program, Primer3 and checked in the BLAST database for any homology to other sequences. Oligonucleotides were obtained from MWG Biotech and on arrival they were initially diluted to 100 pmol per μ l and further diluted to 30 pmol per μ l before forwarding to DBS Genomics for further sequencing of the *V. splendidus* mutant DNA inserts.

5.2.14. Sequence analysis

The DNA sequences were received from DBS Genomics via email and sequences were analysed and aligned using the GeneBuilder sequence analysis program. The

sequences were identified using the EBI website with BLAST and FASTA databases searches.

5.2.15. Determination of potential virulence determinants in transposon mutants

5.2.15.1. Haemolysin assay

Haemolysin production by transposon mutants was measured using the haemolysin assay as previously described (section 2.2.8 and 3.2.2).

5.2.15.2. Tissue culture assays

The tissue culture cell line TV1-S4 was used as described in chapter 3 (section 3.2.5) to assess the cytotoxicity activity of the haemolysin-negative mutants compared to the parent DMC-1 strain. Tissue culture cells were treated with filter-sterilised culture supernatant and washed bacterial cells from 24 h cultures. The bacterial cells were washed in reduced NaCl conditions using MEM as a buffer with successive washes at salt concentrations of 1.5 %, 0.75 % and 0.9 %. The washed bacterial cells were inoculated into tissue culture plate wells in duplicate at 1×10^3 and 1×10^7 cfu ml⁻¹. The filter-sterilised supernatant was added to wells at a 1/10 dilution in MEM. Plates were incubated at 20 °C and cells were assessed visually for cytopathic effects using an inverted microscope. The plates were inspected every hour for 4 h and then after 24 h.

5.2.16. *In vivo* assays

5.2.16.1. Yolk-sac larval trials 1 & 2 -Bergen, Norway

The protocol in chapter 3 (section 3.2.6) for the yolk-sac turbot larval challenges was followed with the addition of two haemolysin-negative mutants, *V. splendidus* DMC-1-M2 and M3. Both these strains were grown under the same conditions as *V. splendidus* DMC-1.

5.2.16.2. First-feeding larval trial -Bergen, Norway

The protocol described in Chapter 3 (section 3.2.7) for the first-feeding trial was followed with the addition of another group containing the haemolysin-negative mutant DMC-1-M2. The *V. splendidus* DMC-1-M2 strain was grown under the same

conditions as the *V. splendidus* DMC-1 strain. The concentration of DMC-1-M2 was 7.5×10^7 and 4.4×10^7 cfu ml⁻¹, on each challenge day. Immunohistochemistry was applied to the larvae sampled from the challenge trial as before.

5.2.16.3. First-feeding trial-Glasgow

The first-feeding larval challenge trial protocol from chapter 3 (section 3.2.8) was followed. For the trial the haemolysin-negative mutant *V. splendidus* DMC-1-M4 was grown under the same conditions as *V. splendidus* DMC-1 on a daily basis. The DMC-1-M4 strain was added daily to the rotifers from day 3 until day 9, following the chapter 3 protocol for the addition of DMC-1 to rotifers for days 5-7. The groups challenged with DMC-1-M4 also received a challenge of DMC-1 on days 5-7. The mortalities were measured and bacteriology of larval gut and tank water recorded on day 9, as in chapter 3.

5.3. Results

5.3.1. Haemolysin production

The optimum production of haemolysin by *V. splendidus* DMC-1 occurred between 16 and 24 h as shown in Figure 5.4. The levels of haemolysin in the culture reduced with increasing age of the culture. This may be a result of the bacteria losing viability and thus not producing active haemolysin as occurs during exponential growth. The haemolysin may also be degraded over time by other factors produced by the growing bacteria, for example proteases.

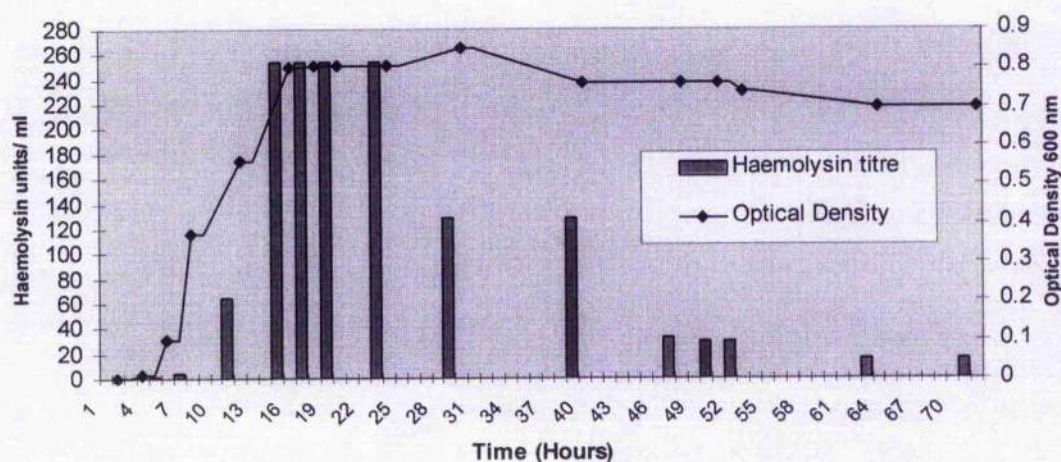


Figure 5.4. Growth of *V. splendidus* DMC-1 and production of haemolysin during a 72 h period. Growth was measured by optical density readings at 600 nm, haemolysin activity was titrated in a haemolysin assay using doubling dilutions of filter-sterilised culture supernatant.

5.3.2. Effect of temperature on binding of haemolysin to erythrocytes

Crude culture supernatant was incubated with varying concentrations of sheep erythrocytes at 0 °C for 1 h. No lysis was observed with any of the mixtures but on resuspension of the pellet of blood in PBS, haemolysis occurred. Therefore, it appeared that the toxin binds to the cells at 0 °C and does not cause lysis until the temperature is raised. This suggests that haemolysis is temperature-dependent but binding to the cell membrane occurs independently of temperature.

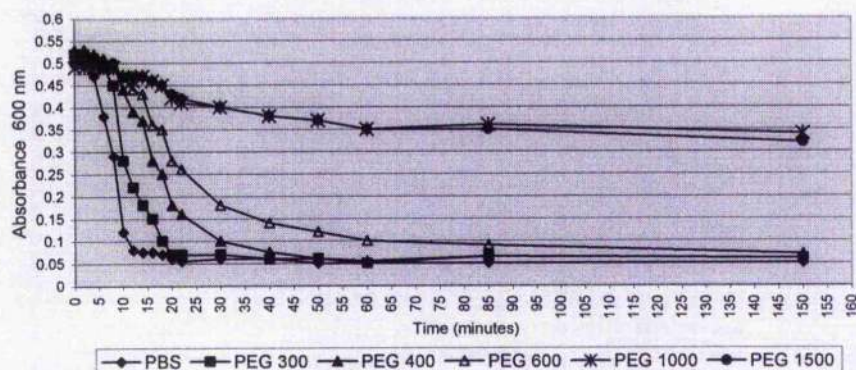
5.3.3. Concentration of haemolysin

Haemolysin from the *V. splendidus* DMC-1 culture supernatant was concentrated successfully with the Filtron 10K filter system and the haemolytic activity increased 8-fold from 1/128 in 2 L to 1/1024 in 100 ml. Overall, 40 % of the activity was recovered. In further experiments the system was used to concentrate 4 L, which was reduced to 90 ml and gave a haemolytic titre of 1/4096. This method was considered satisfactory for concentration of large volumes of culture supernatant.

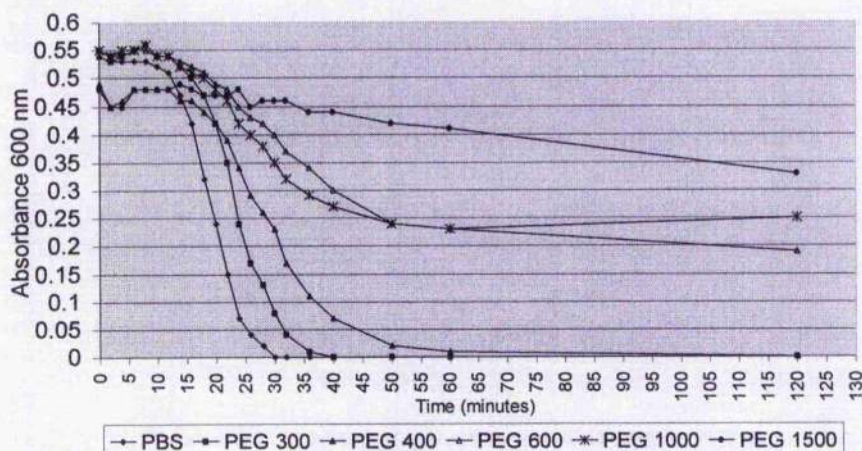
5.3.4. Osmotic protection studies

To determine whether the mechanism of lysis of erythrocytes was by colloid osmotic lysis the erythrocytes were exposed to haemolysin in the presence of a range of solutes of increasing viscometric radius. If the haemolysin forms transmembrane pores or channels, solutes and water may equilibrate inside and outside of the membrane. If the solutes are too large to pass through the pore, e.g. intracellular proteins, osmotic imbalance results such that water flows into the cell through the pores causing cells to swell and lyse. In these studies blood cells were suspended in different solutions of polyethylene glycols with and without added toxin. If the polyethylene glycol was too large to pass through the pores in the cell membrane, cells were osmotically protected and did not lyse. If the polyethylene glycols were able to pass through the pores, they would be unable to prevent osmotic imbalance and cells would lyse. The kinetics of this lysis for 3 separate experiments are shown in Figure 5.5.

5.5a Experiment 1



5.5b Experiment 2



5.5c Experiment 3

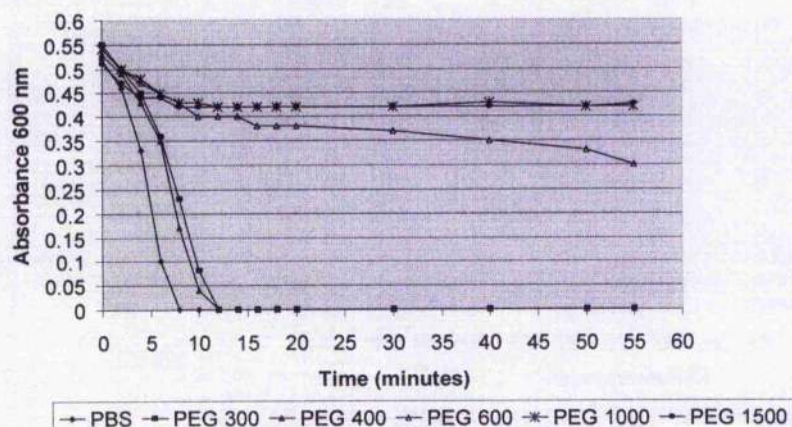


Figure 5.5. Osmotic protection studies of erythrocytes exposed to concentrated culture supernatant of *V. splendidus*. Three experiments were carried out 5.5a-c. Toxin-treated cells were suspended in solutions of PEG with different molecular sizes and haemolytic activity was measured using cell scattering data at an absorbance of 600 nm. Toxin-treated cells with PBS added were used as positive haemolytic controls.

Time taken for 50 % lysis to occur was determined graphically and the data from the osmotic protection experiments for each polyethylene glycol was plotted against the known viscometric radius of the glycols (Table 5.1) to enable estimation of the size of pore formed by the toxin (Figure 5.6). Extrapolation of the lines in this graph gave an estimated pore size of approximately 1.8-1.9 nm in diameter.

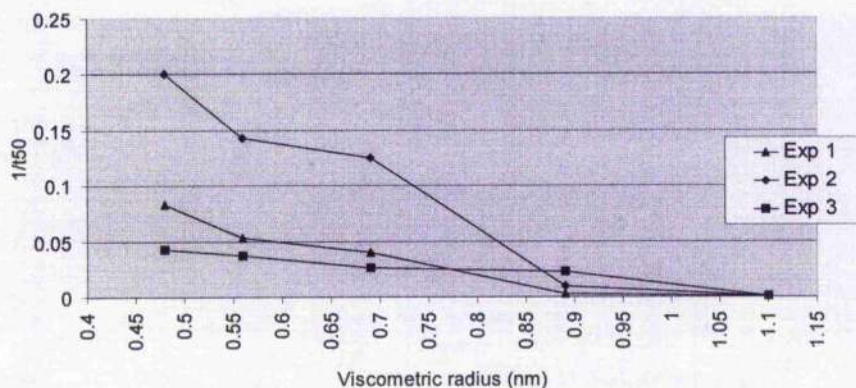


Figure 5.6. Estimation of transmembrane-pore diameter in erythrocyte membranes treated with concentrated culture supernatant of *V. splendidus* DMC-1. The $1/t_{50}$ data from figure 5.5a-c was plotted against the known viscometric radii of the PEG solutions (Table 5.1) for all three experiments. The estimated radius of the pore formed by the *V. splendidus* toxin can be extrapolated from these results to be ~0.9 nm.

The effect of metal ions and inhibitors on haemolysis was assessed by measuring the kinetics of haemolysis over a period of time with the solutions added to blood cells treated with the toxin. Table 5.3 gives an overview of both the metal ions and the inhibitor solutions at fixed concentrations and it also shows the overall percentage of inhibition. Only manganese sulphate and zinc sulphate inhibited the haemolytic action of the toxin and further experiments were carried out using these metal ion solutions at varying concentrations (Figure 5.7). However, manganese sulphate did not inhibit lysis at any of the concentrations tested in this experiment but the zinc sulphate inhibited the haemolytic action at 1 and 5 mM.

Material tested	Haemolysin titre	Haemolysin activity (% of control)	Inhibition (%)
Crude haemolysin ¹	1/1024	100	0
CaCl ₂ + haemolysin ¹	1/1024	100	0
CaCl ₂	- ²	-	-
MgSO ₄ + haemolysin	1/1024	100	0
MgSO ₄	-	-	-
MnSO ₄ + haemolysin	1/128	12.5	87.5
MnSO ₄	-	-	-
ZnSO ₄ + haemolysin	1/256	25	75
ZnSO ₄	-	-	-
EDTA + haemolysin	1/2048	200	0
EDTA	-	-	-
DTT + haemolysin	1/1024	100	0
DTT	-	-	-

Table 5.3. Effect of metal ions and inhibitors on the haemolytic action of the *V. splendidus* DMC-1 toxin. Haemolytic activity was titrated in a haemolysin assay using doubling dilutions. ¹Concentrated culture supernatant of *V. splendidus* DMC-1 was used as a positive haemolytic control. All metal ions and inhibitors were tested against erythrocytes as negative controls. ²Negative for haemolytic activity.

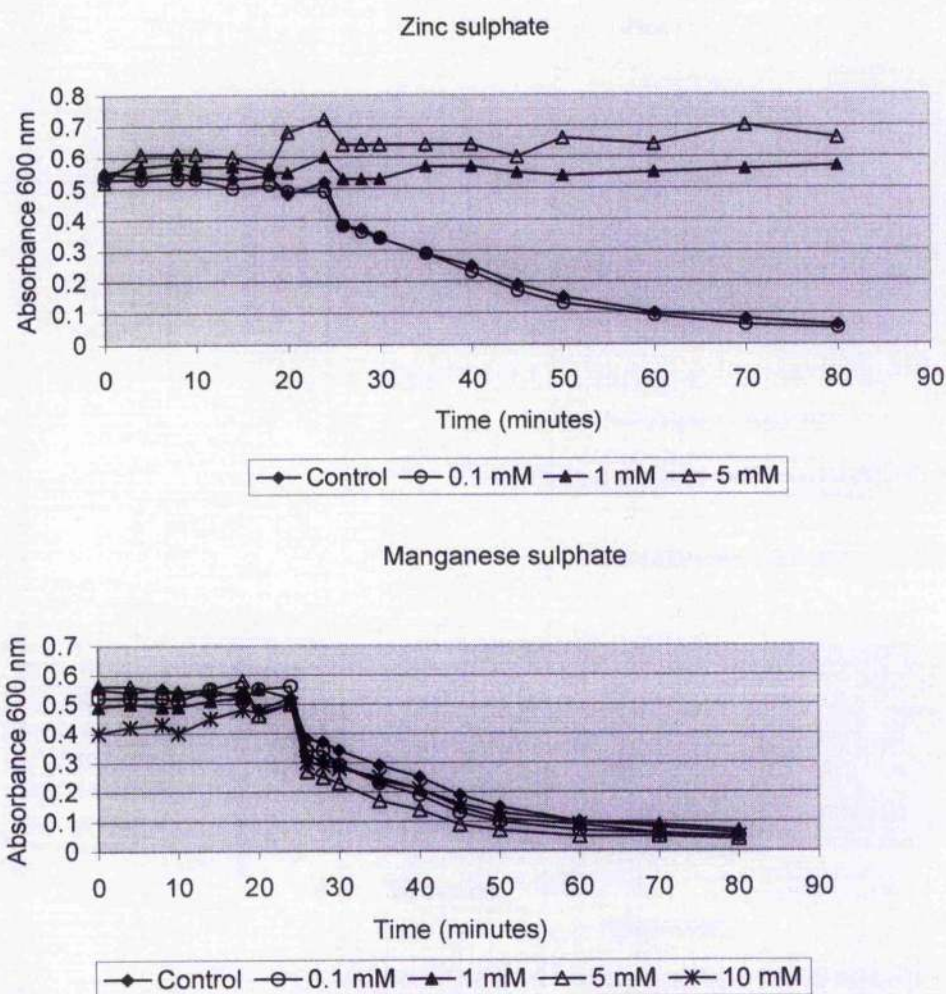


Figure 5.7. Effect of metal ions, zinc sulphate (concentration 0.1, 1, 5 mM) and manganese sulphate (concentration 0.1, 1, 5, 10 mM) on the haemolytic activity of filter-sterilised bacterial supernatant of *V. splendidus* DMC-1 against 1 % sheep red blood cells. The positive haemolytic control contained filter-sterilised bacterial supernatant. Haemolytic activity was measured using absorbance readings at 600 nm.

5.3.5. Purification of *V. splendidus* cytotoxin by column chromatography

5.3.5.1. Ion-exchange chromatography

The HiTrap ion-exchange mini columns were used to assess the best matrix for purification of the toxic factor prior to running larger scale ion-exchange columns. Overall, the matrix that had the least effect on the activity was a strong anionic exchanger that allowed the toxin to pass directly through the column whilst binding a high amount of other proteins from the concentrated supernatant. This identified the

toxin as being positively charged with the majority of contaminating proteins being negatively charged. The effects of the four types of material are shown in Table 5.4. When the toxin bound to the cationic columns and was then eluted, very little activity was recovered.

Exchanger	% Recovery of active material
CM (Carboxymethyl) Cationic exchanger	6
SP (Sulphopropyl) Cationic exchanger	0.6-1.25
DEAE (Diethylaminoethyl) Anionic exchanger	4-20
Q (Quaternary ammonium) Anionic exchanger	50

Table 5.4. Efficiency of different ion-exchange column material for the purification of haemolytic factor(s) from *V. splendidus* DMC-1 bacterial supernatant.

The Q-column was scaled up to enable a larger volume of culture supernatant to be applied to the column and the recovery of haemolytic activity and protein concentration of the fractions from the column are shown in Figure 5.8. The active haemolytic material was recovered between fractions 4 and 10, with a corresponding peak of protein between 50 and 150 $\mu\text{g ml}^{-1}$. Higher concentrations of protein occurred in the later fractions when the column was eluted with buffer containing NaCl. Therefore, the majority of contaminating protein was removed efficiently with this column resulting in significant purification of the haemolytic factor(s).

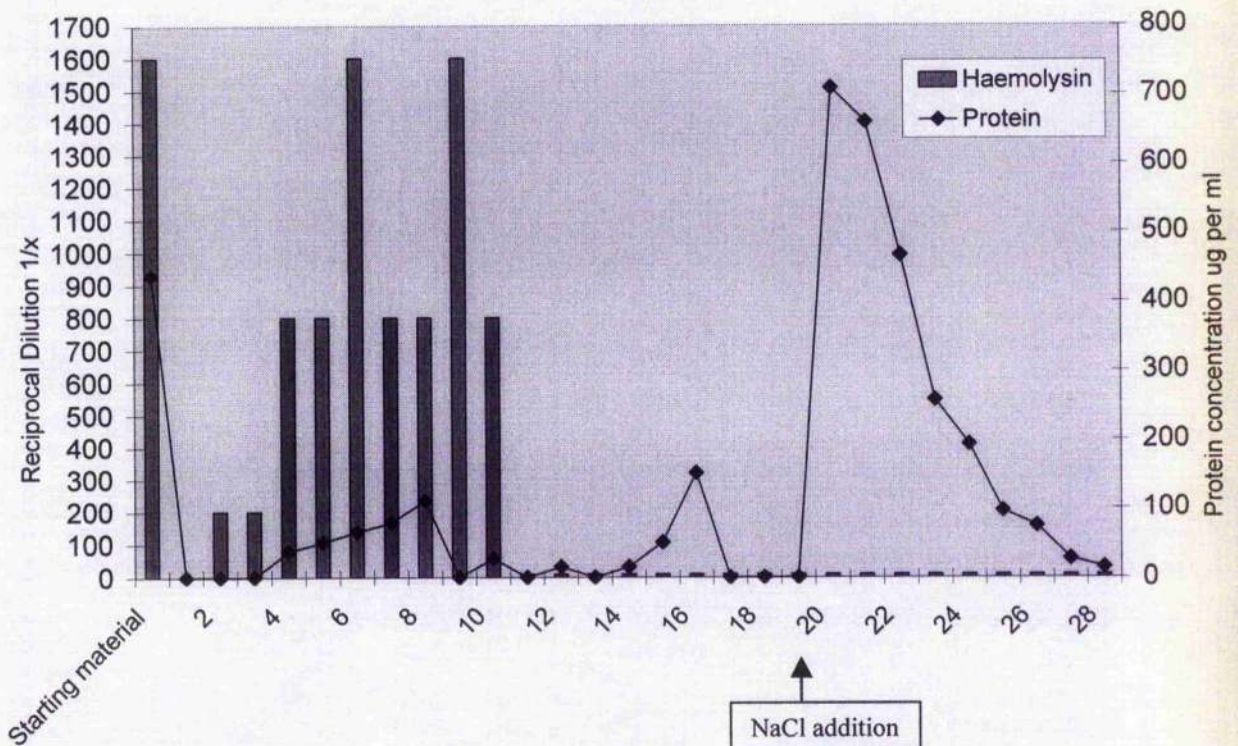


Figure 5.8. Haemolytic activity and protein concentration of fractions 1-28, eluted from ion-exchange quaternary ammonium sepharose column (Q-column). *Vibrio splendidus* bacterial supernatant was included as a positive control.

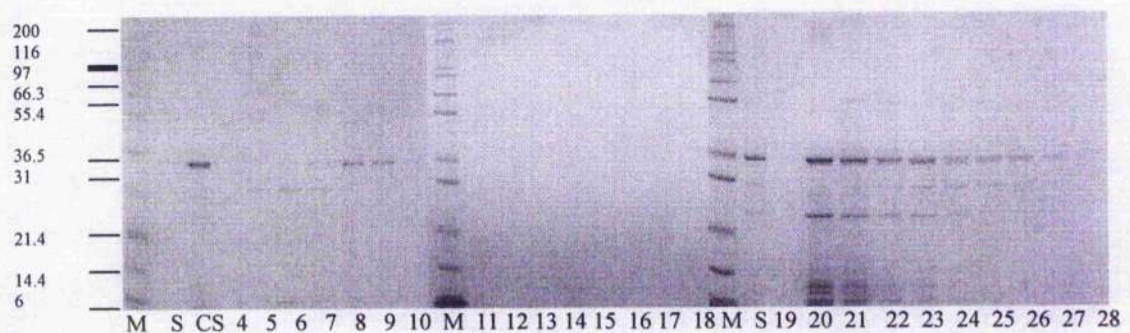


Figure 5.9. Protein banding in SDS-PAGE gels containing eluted fractions (4-28) from ion-exchange Q-column and culture supernatant of *V. splendidus* DMC-1. M= Mark12 standard, S= Supernatant, CS= Concentrated supernatant. Protein standard, Mark 12 marker was used in lane 1 of each gel with sizes in kD indicated at the left. Gels were stained with Coomassie Brilliant Blue stain.

The gels in Figure 5.9 show the protein bands present in the fractions from the Q-column. Fractions 4 to 10 in the first gel contain the active toxin. Faint bands were present at 32 and 35 kD, with the 35 kD protein band being dominant in the concentrated culture supernatant. Fractions 11 to 19 were collected after washing the column with 20 mM Bis-Tris buffer and fractions 20 to 28 were eluted from the column with the same buffer containing 0.4 M NaCl. There are no bands visible in fractions 11 to 19, and proteins attaching to the column are eluted in fractions 20 to 27. The protein band at 35 kD is present in all of these fractions and the 32 kD protein is present in fractions 22 to 27. Therefore, there is no obvious protein band identified as the haemolysin. These SDS-PAGE gels do show that the majority of the protein in the supernatant of *V. splendidus* DMC-1 attaches to the column and is eluted using increased concentrations of NaCl as shown in Figure 5.8.

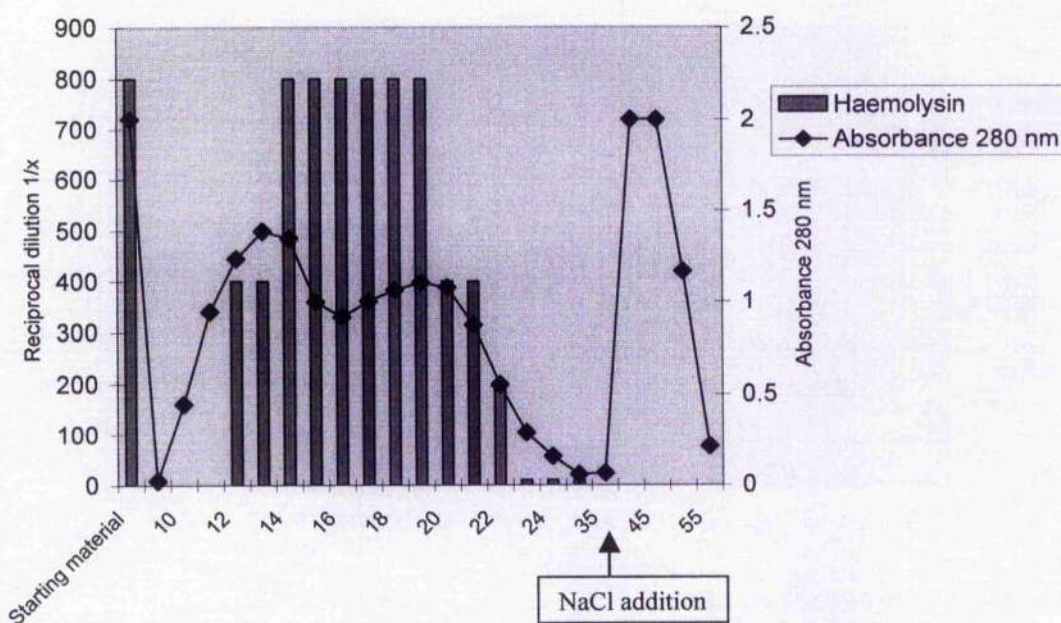


Figure 5.10. Haemolytic activity and estimated protein concentration of fractions 9-25, 35, 40, 45, 50, 55 eluted from ion-exchange quaternary ammonium sepharose column (Q-column). *Vibrio splendidus* DMC-1 bacterial supernatant was included as a positive control.

A second column gave similar results as shown in Figure 5.10. The active fractions (12 to 22) from this column were pooled and used for further analysis. The later

fractions (40 to 44) contained the majority of the protein from the supernatant and required salt to elute them from the column; these were used as a comparison.

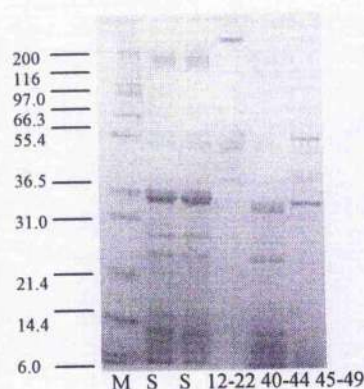


Figure 5.11. SDS-PAGE gel showing protein bands of pooled fractions 12-22, 40-44 and 45-49 from an ion-exchange Q-column alongside original bacterial supernatant (S) of *V. splendidus* DMC-1. Protein standards used was Mark12 (M) with band sizes indicated at the left in kD.

Figure 5.11. shows the difference in the banding patterns of the proteins between the pooled fractions compared to the active starting material. In this experiment there was more protein present in the starting material, resulting in additional bands seen in the fractions from the column. The protein bands within fractions 12 to 22 were of 12, 32, 40, 50, 55, 62, 78 and >200 kD. There was no band at 35 kD as seen in the previous experiment. As this 35 kD protein was present in the later eluted fractions of the previous experiment, it suggests that Figure 5.11 shows a more highly purified preparation of the toxin. The later fractions show the different proteins that have attached to the column. Similarities between the active fractions were in the proteins at 40 kD and >200 kD. This protein band at >200 kD did not appear in the starting material which may indicate that oligomerisation of a protein has occurred, perhaps as a result of purification of such a protein away from other proteins thus allowing better interaction between the monomers.

To assess if oligomers were responsible for the high molecular weight protein band, the pooled active fractions were heat-treated and analysed by SDS-PAGE. This was

to allow the >200 kD protein to dissociate into monomers and possibly become denatured. The banding pattern shown in Figure 5.12 was as found in the same samples shown in Figure 5.11 with only a few minor differences. These differences were that additional bands were present in the 70 °C and 100 °C heated samples. An additional band at 53 kD was present and in the 100 °C samples triple bands at >200 kD were seen, as well as a faint band at 32 kD. At the higher temperatures the protein present at >200 kD may have dissociated to produce smaller protein bands. Therefore, the >200 kD protein could represent an oligomer of an active toxin.

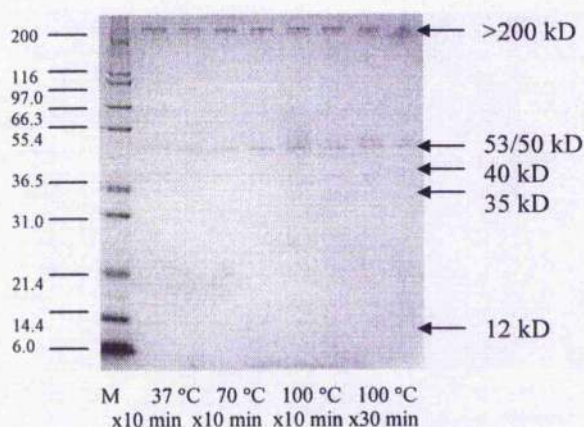


Figure 5.12. Analysis of heat-treated fractions (12-22) from ion-exchange Q-column using SDS-PAGE gel. The heat treatments were 37 °C x 10 min, 70 °C x 10 min, 100 °C x 10 min and 100 °C x 30 min in duplicate. Protein band sizes are indicated with arrows to the right of the gel. The protein standard, Mark12 (M) was used with the sizes indicated at the left in kD.

The pooled fractions 12 to 22 containing the haemolytic activity were screened using a native polyacrylamide gel to enable the haemolysin to remain active. A comparison of the gel with sheep blood agarose overlay and silver-stained bands is shown in Figure 5.13. The first gel shows a clear zone in the blood overlay indicating the presence of the active haemolysin. This zone corresponds with a separate gel stained with silver stain that contained the same fractions from the Q-column.

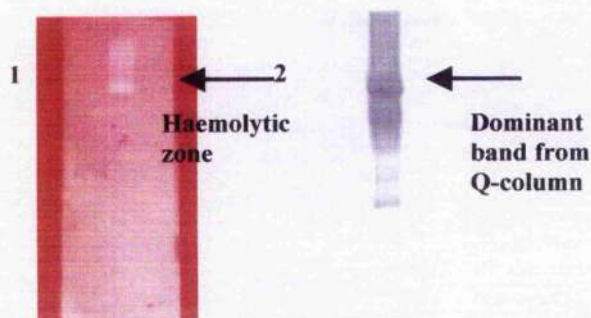


Figure 5.13. Native PAGE gels containing haemolytic fractions from ion-exchange chromatography column (Q-sepharose). Fractions were run in duplicate gels and treated with 1. blood overlay and 2. Silver stain. Arrows indicate the haemolytic zone and a corresponding dominant band in silver stained gel.

Therefore, the haemolytic toxin binds to the cationic CM and SD HiTrap columns but not to the anionic exchangers, DEAE and Q columns and is a positively-charged protein. The Q-sepharose allowed purification of the haemolysin from more negatively-charged proteins from the supernatant. However, this method of purification did not result in a homogenous preparation of the haemolysin as several protein bands were present apart from that corresponding to the band of haemolytic activity. Further purification was attempted by pooling together the active fractions from this column and applying them to a different type of chromatography column.

5.3.5.2. Hydrophobic interaction chromatography

The pooled fractions from the Q-column were used as the starting material for the phenyl-Sepharose column chromatography. Initially, a linear gradient of decreasing ammonium sulphate and increasing polyethylene glycol (final concentration 0 and 80 %, respectively) was applied using a gradient mixer. However the activity of the toxin was lost under these conditions (data not shown). Ranges of ammonium sulphate and polyethylene glycol concentrations were tested to allow the active material to attach to the column and then be eluted off. However, the active material was either lost under these conditions or the material remained attached to the column, suggesting that this protein is hydrophobic. The conditions were changed to allow the active material to pass directly through the column as in the ion-exchange chromatography column. By adding 10 % ammonium sulphate directly to the 0.05 M Tris HCl buffer, the active material passed through the column. As shown in Figure

5.14 the active material is associated with the majority of the protein, unlike the Q-column (Figures 5.5 and 5.7). However, the specific activity of the material dropped eight-fold during this purification step.

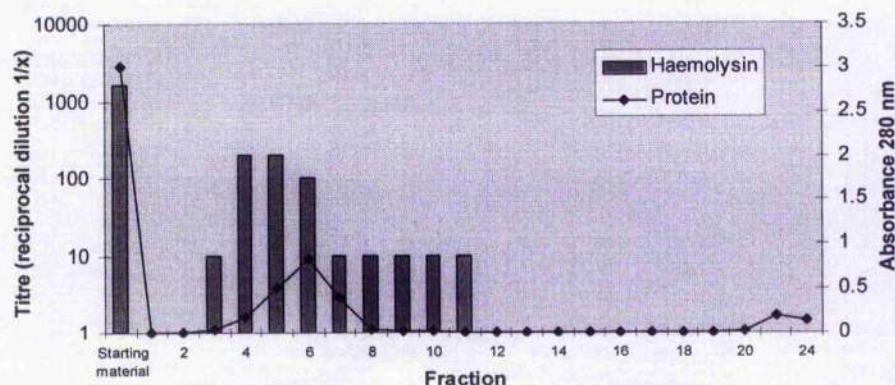


Figure 5.14. Protein concentration and haemolytic activity of fractions (1-24) from hydrophobic interaction chromatography column.

The pooled active fractions were analysed on a native polyacrylamide gel but no activity could be detected with the blood overlay on the gel. The native gel was also stained with Coomassie blue and when compared to the native gel with blood overlay for the active Q-column fraction, the dominant band corresponded to the zone of haemolysis (Figure 5.15) suggesting that the purified material although it had lost a lot of activity, is the haemolysin. However, hydrophobic interaction chromatography was not a successful purification step as shown in Table 5.5, the overall yield of activity was only 3.0 % compared to 20 % from the Q-column.

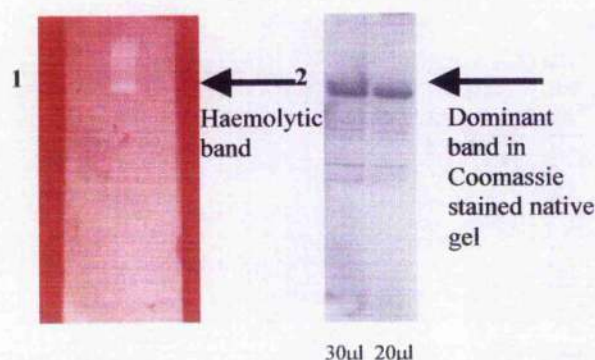


Figure 5.15. Native PAGE tris-glycine gels of haemolytic fractions separated by two types of column chromatography. **1.** Fractions eluted from ion-exchange Q-sepharose column, and overlaid with blood. **2.** Fractions eluted from hydrophobic interaction phenyl-sepharose column, and stained with Coomassie Brilliant blue stain.

Material	Volume (ml)	Total protein (mg)	Haemolysin titre (HU) ml ⁻¹	Specific activity (HU/mg)	% Activity Yield
Concentrated supernatant	10	13.47	16000	1187.8	100
Dialysed supernatant	10	10.26	16000	1559.5	100
Q-column fractions 12-16	9	1.35	3600	2666.7	23
Concentrated Q-column fractions 12-16	3	0.78	4800	6153.9	30
Phenyl-sepharose fractions 4-6	4.5	* ¹	450	*	3
Concentrated phenyl-sepharose fractions 4-6	2	0.132	200	1515.2	1.25

Table 5.5. Haemolytic activity in fractions from both ion-exchange and hydrophobic interaction chromatography compared to the original supernatant. ¹No data, measurements not taken.

The fractions from both columns were compared by SDS-PAGE to assess the banding patterns (Figure 5.16). As found previously, the concentrated Q-column fractions produced a dominant band at >200 kD, a further band of c.50 kD and a faint band at 40 kD, whereas the bands from the phenyl-sepharose fractions are at 50 kD, 29 kD and 8 kD. The lack of protein bands at >200 kD and 40 kD in the phenyl-sepharose column fractions suggests that these are involved in haemolysis and that their levels have been reduced by this type of purification. These bands were not dominant in the

original concentrated supernatant suggesting that the purification with the Q-column had concentrated them to higher levels so that they were detectable in SDS-PAGE.

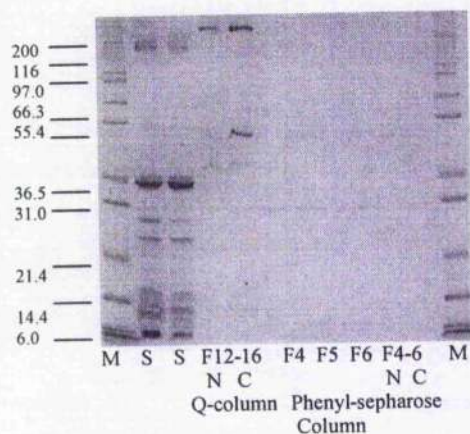


Figure 5.16. SDS-PAGE analysis of haemolytic fractions from ion-exchange (Q-column) and hydrophobic interaction chromatography (Phenyl-sepharose column). Gel was stained with Coomassie Brilliant Blue stain. M, Mark 12 marker (kD); S, Supernatant; N, Native supernatant; C, Concentrated supernatant.

5.3.5.3. Molecular weight exclusion chromatography.

Initial experiments with Sephacryl S-200 produced fractions with poor haemolytic activity and this was thought to be due to the protein binding to the molecular weight substrate, or to the glass column. To reduce such binding a protein carrier, BSA was added to the starting material prior to loading onto the column. The BSA was also added to the elution buffer and the first 45 ml eluted from the column was allowed to flow to waste before collection of fractions.

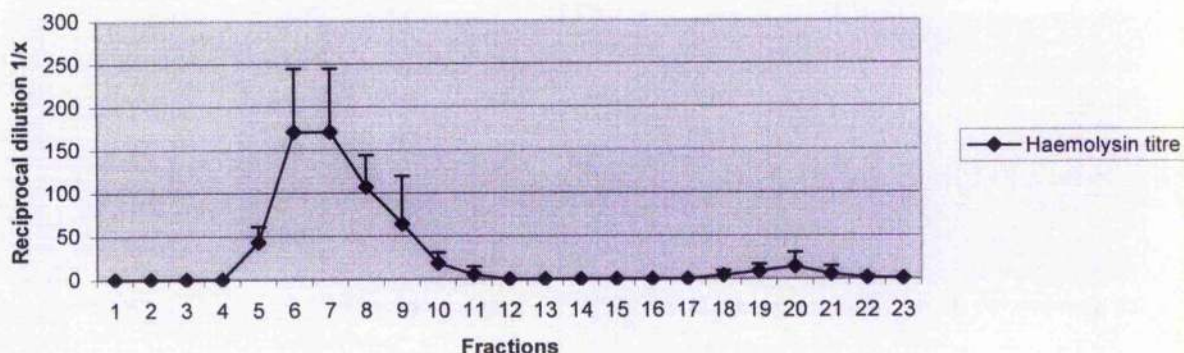


Figure 5.17. Haemolytic activity in fractions from molecular weight exclusion chromatography. Standard deviations are shown from three experiments.

The haemolysin titre of the fractions was measured in three experiments and the averages with the standard deviations are shown for each fraction in Figure 5.17. A small peak of activity was also found in the later fractions, 18 to 21, suggesting either the presence of two distinct haemolysins or could represent a monomer and oligomer. No protein measurements were recorded from the latter gel filtration experiments due to the presence of a high concentration of BSA carrier protein.

The active haemolytic fractions from the sephacryl column were analysed on SDS-PAGE, with no BSA added (Figure 5.18), and bands at >200 kD and 48-50 kD were present as was found for the Q-column fractions. Other bands present were of 64 kD, 60 kD, 53 kD, 52 kD and 40 kD. Pooled fractions 18 to 23 were also included, but no corresponding bands were found between the two sets of fractions. As noted above, the activity in the later fractions may be monomeric toxin that has disassociated from the oligomeric form present in the earlier fractions.

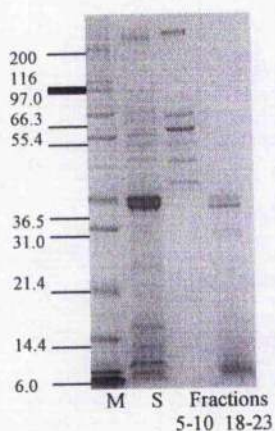


Figure 5.18. Analysis of pooled haemolytic fractions (5-10 and 18-23) from molecular weight exclusion chromatography with no BSA added (Sephacryl S-200) using SDS-PAGE. Gel was stained with Coomassie Brilliant Blue stain. M, Mark 12 marker (kD); S, supernatant.

5.3.6. Isoelectric focusing.

The concentrated supernatant from the Filtron system was applied to an IEF gel as an alternative method for purification of the haemolysin. However, little of the haemolytic activity was recovered (Figure 5.19) suggesting that the haemolysin may be losing co-factors necessary for activity when proteins in the supernatant are separated according to their isoelectric points (pI). Evidence for this is shown in Figure 5.19, where low haemolytic activity is detected in fractions 7-9, 13-16 and 20 as well as the detection of high activity in fractions 17-20. The pI of the haemolysin, based on the proteins with highest activity, is 6.5-6.9. However, this may not be an accurate estimate as other fractions are haemolytic.

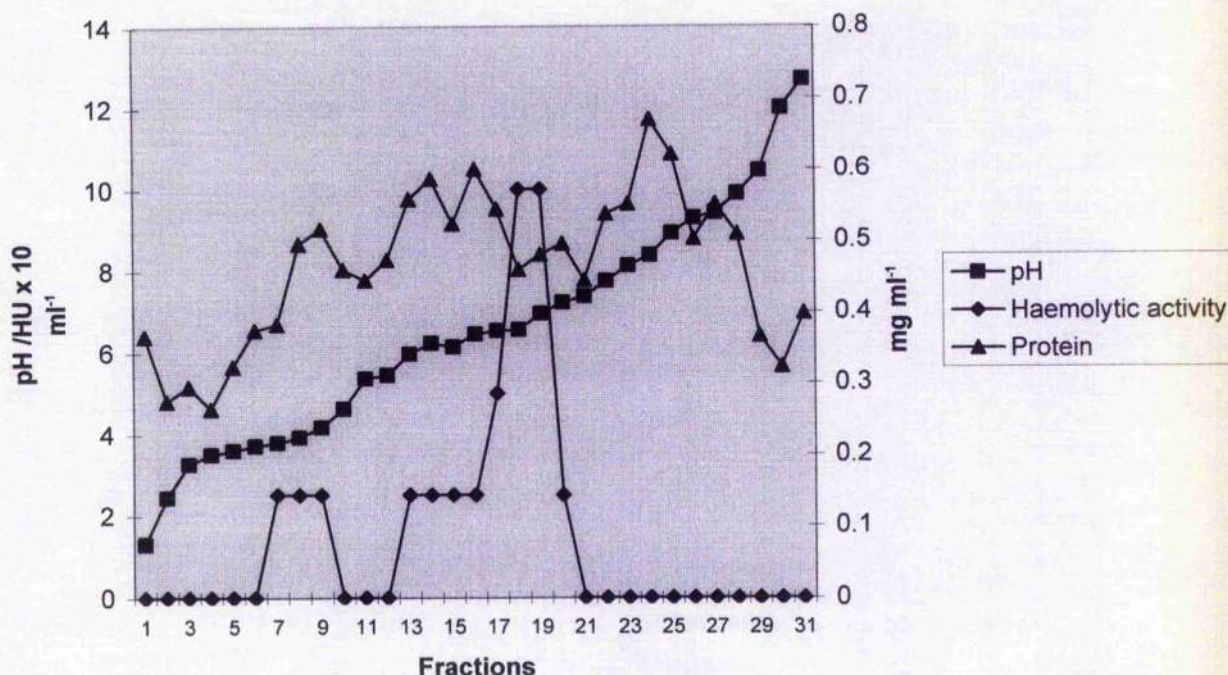


Figure 5.19. Measurement of the pH, protein and haemolytic activity of fractions from an isoelectric focusing (IEF) gel. The starting material on the IEF gel was concentrated *Vibrio splendidus* DMC-1 supernatant.

5.3.7. Molecular Methods for identification of the haemolysin

5.3.7.1. Genomic DNA plasmid library

The genomic DNA from *V. splendidus* was digested with the restriction enzyme *Sau3A* to produce fragments compatible with *Bam*HI-digested pUC18 vector (Figure 5.20). In initial work a digestion series of the *Sau3A* enzyme was used to obtain fragments in the size range of 2-4 kb. Fragments of the correct size were ligated into the plasmid vector and resulting plasmids were analysed to determine the average size of the DNA insert before screening for haemolytic activity.

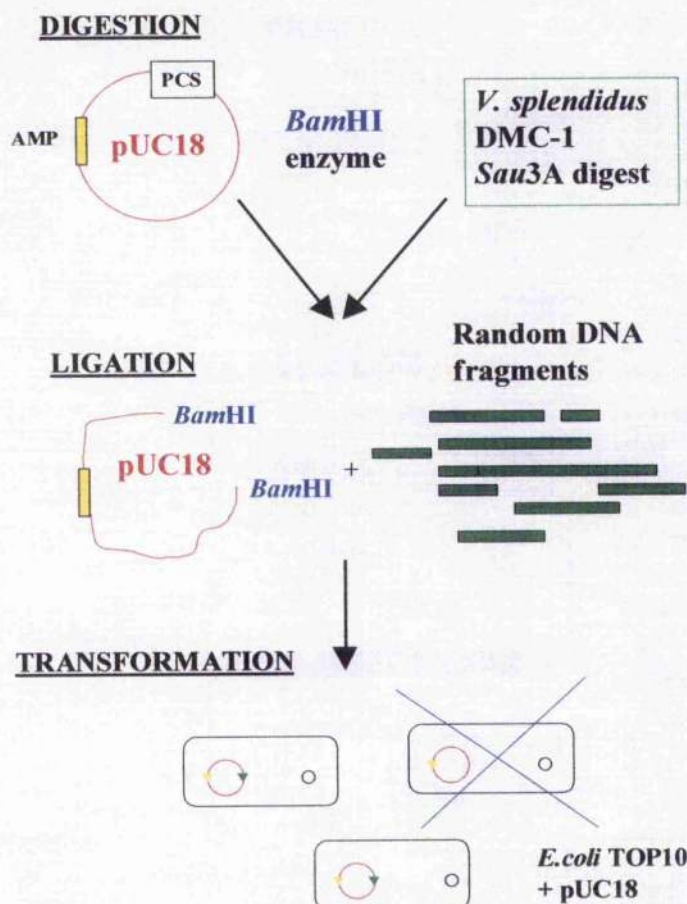


Figure 5.20. Overview of genomic plasmid library construction. Plasmid vector pUC18 was digested with *Bam*HI giving ends that were compatible with *Sau*3A digested *V. splendidus* DMC-1 genomic DNA fragments. Plasmid clones containing inserted DNA were selected for further analysis.

As shown in Figure 5.21 the average DNA insert in this plasmid library was 2.5 kb, which was thought to be too small to provide coverage of the haemolysin gene. The digestion series was repeated to obtain fragments larger than 4 kb. In this way the average insert size obtained was increased to 3.9 kb.

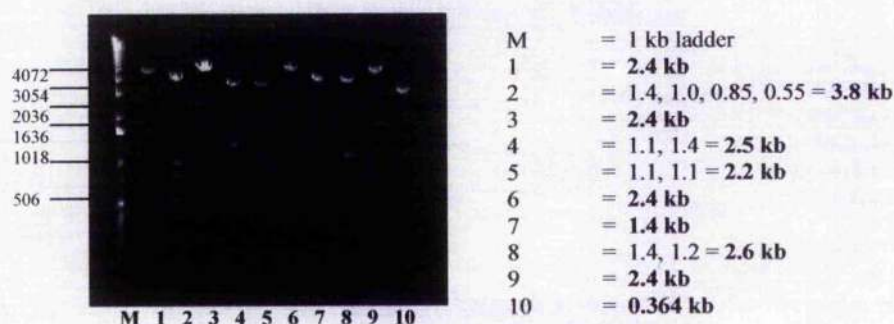


Figure 5.21. Plasmid digestions with *Eco*RI of pUC18 vector containing 2-4 kb ligated fragments of *V. splendidus* DMC-1 DNA. The total DNA insert size for each clone is highlighted in bold text.

Both sets of libraries created were screened for haemolytic clones on selective blood plates. One haemolytic clone was identified from the first library and three from the second. The activity of two of these clones is shown in Figure 5.22.

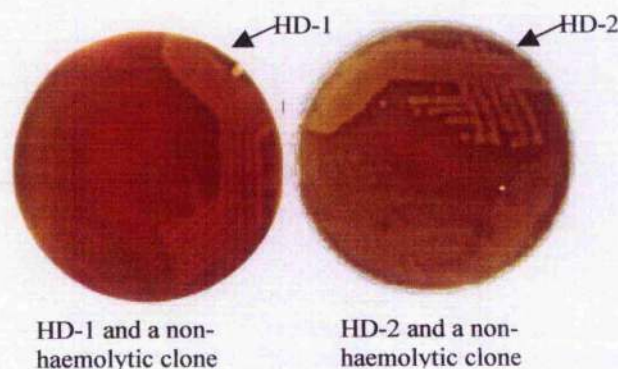


Figure 5.22. Haemolytic plasmid DNA library clones, HD-1 and HD-2 alongside a non-haemolytic clone. Haemolytic clones are indicated with arrows. Plasmids are contained in *E. coli* TOP10 cells.

5.3.7.2. Analysis of haemolytic clones

The DNA inserts of the haemolytic clones were sequenced to determine 600 bp from each end. These sequences were entered into the EMBL database and matches obtained via BLAST searches. To obtain further DNA sequence, the DNA fragments contained within the pUC18 vectors were subjected to restriction enzyme digestion to

reduce the size of the insert for further sequencing (Figure 5.2). The fragments obtained from these digests were analysed enabling the location of a number of restriction sites within the DNA insert based on the banding patterns produced (Figure 5.23).

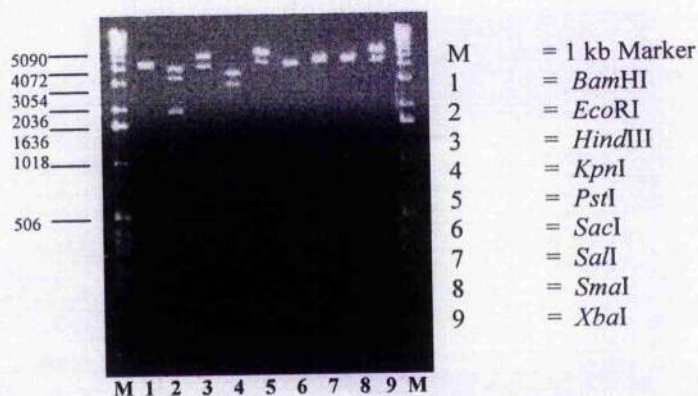


Figure 5.23. Haemolytic plasmid clone, HD-1 (pUC18 plasmid + *V. splendidus* DMC-1 DNA insert) digested with a number of restriction enzymes. Restriction enzymes used are listed at the side of the gel corresponding to the lanes on the gel.

This enabled HD-1 to be truncated into smaller fragments based on the enzyme sites of the insert's restriction map (Figure 5.24A). The fragments obtained were religated and screened for haemolytic activity to produce clones HD-4 and HD-6 (Figure 5.24A). The same strategy was used to produce restriction digest maps of the DNA inserts of all haemolytic plasmid clones (Figure 5.24A-C) and further sequencing of the truncated haemolytic DNA inserts was carried out. Truncated clones obtained from clone HD-3 were HD-7, 8 and 9 (Figure 5.24C).

An overview of the haemolytic clones created in the plasmid library is shown in Table 5.6. The DNA inserts were sequenced (Table 5.6) and their closest match was obtained using the BLAST database of EMBL. The sequence matches are shown in Table 5.7. Overall, there were no specific matches to haemolysin genes in any of the haemolytic plasmid clones identified.

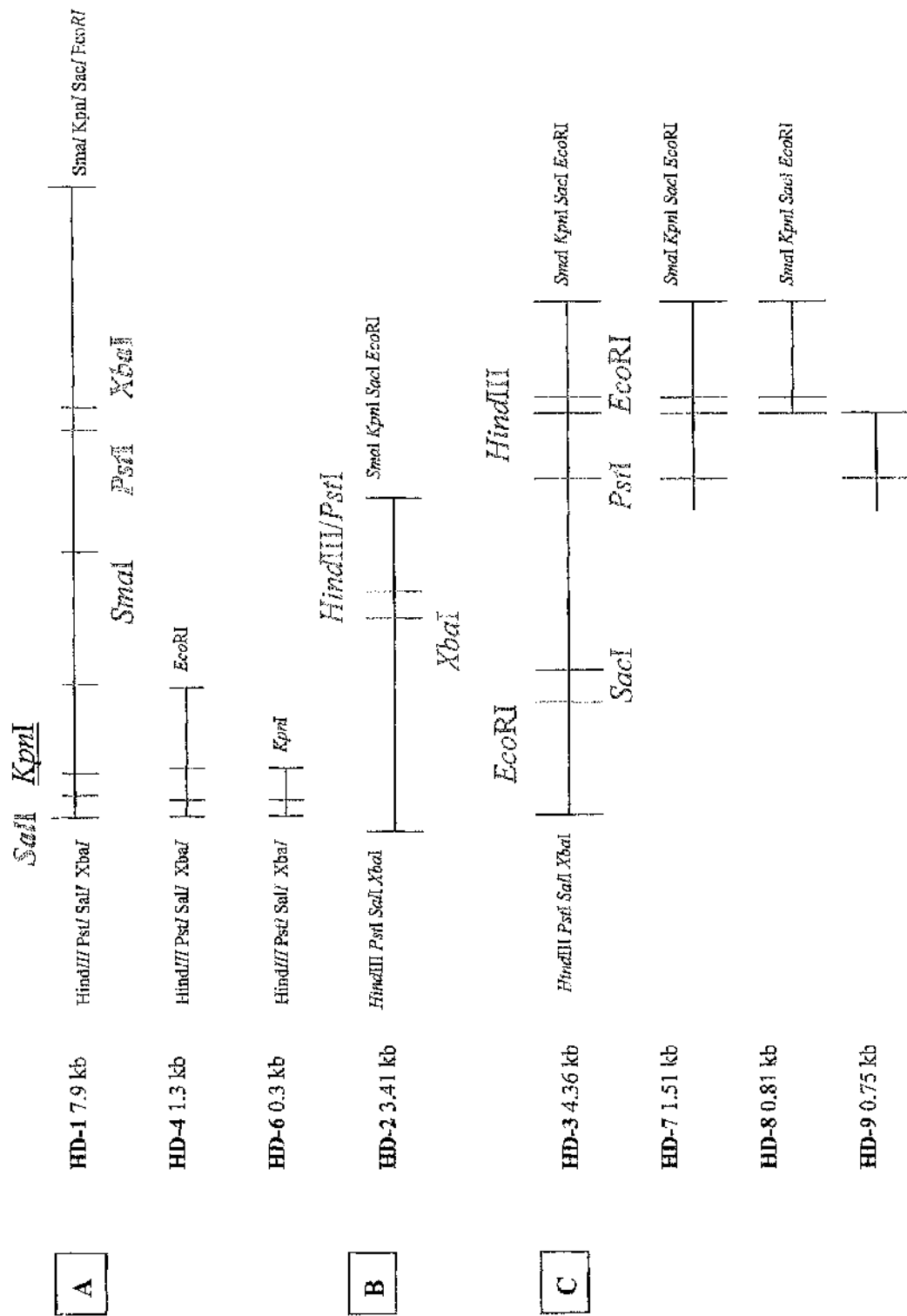


Figure 5.24. Restriction enzyme maps for haemolytic plasmid clones HD-1 to HD-9. **A.** HD-1 insert with truncated inserts HD-4 and HD-6; **B.** HD-2; **C.** HD-3 with truncated inserts of HD-7, 8 and 9. Inserts in A and C were truncated using the appropriate restriction enzymes whilst retaining haemolytic activity.

Clone	DNA insert size (kb)	Origin	Sequencing
HD-1	7.9	2-4 kb library original clone	M13 forward and reverse
HD-2	3.41	>4 kb library original clone	M13 forward and reverse
HD-3	4.36	2-4 kb library original clone	M13 forward and reverse
HD-4	1.3	Religation of 4.0 kb <i>Eco</i> RI HD-1 fragment	M13 reverse
HD-5	3.41	>4 kb library original clone	Not done
HD-6	0.3	Religation of 0.3 kb <i>Kpn</i> I HD-1 fragment	Covered by M13 forward in HD-1
HD-7	1.51	Religation of 4.3 kb <i>Sal</i> I HD-3 fragment	M13 forward
HD-8	0.81	Ligation of 0.81 kb <i>Kpn</i> I/ <i>Hind</i> III HD-3 fragment into new pUC18 vector	M13 forward
HD-9	0.75	Ligation of 0.75 kb <i>Sal</i> I/ <i>Hind</i> III HD-7 fragment into new pUC18 vector	M13 forward and reverse

Table 5.6. Insert size, origin and sequencing primers used for DNA inserts of haemolytic clones (HD-1 to HD-9) from plasmid genomic library.

5.3.7.2. Sequencing results of haemolytic clones

The sequence data is shown in sequencing Appendix 3.

As there was neither a consistent pattern to the fragments found in haemolytic clones (Table 5.7) nor any specific matches to known haemolysin genes within the *Vibrio* genus it was questioned whether the haemolytic clones were identifying the true genes that are responsible for haemolytic activity. The inducing compound IPTG is normally included in the selective plates to induce expression of the genes inserted into the plasmid vector. To assess whether IPTG had an effect on haemolytic activity, selective plates with and without IPTG were inoculated with the haemolytic clones as shown in Figure 5.25 and Table 5.8. The addition of IPTG did not increase the expression of haemolytic activity even showing a stronger haemolytic reaction in HD-3 when IPTG was not added.

Clone	M13f primer sequencing results		M13r primer sequencing results	
	Identity	Gene	Identity	Gene
HD-1	No matches	-	63% <i>V. cholerae</i> ChrII (78 of 93) ¹	<u>VCA0942</u> hypothetical protein
HD-2	66% <i>V. cholerae</i> ChrI (220 of 251)	<u>VC2424</u> TypeIV pilus assembly protein PilB	68% <i>V. cholerae</i> ChrI (220 of 251)	<u>VC2427</u> , <u>VC2428</u> Conserved hypothetical proteins
HD-3	53% <i>V. cholerae</i> ChrI (187 of 251)	<u>VC2052</u> Cytochrome c-type biogenesis protein	70% <i>V. cholerae</i> ChrI (113 of 251)	<u>VC1264</u> Iron-regulated protein A, putative
HD-4	No matches (from HD-1)	-	54% <i>V. cholerae</i> ChrI (98 of 251)	<u>VC1091</u> Oligopeptide ABC transporter, periplasmic
HD-7	78 % <i>V. cholerae</i> ChrI (113 of 251)	<u>VC1263</u> GTP cyclohydrolase	-	=
HD-8	84/86 % <i>V. cholerae</i> ChrI (113 of 251)	<u>VC1264</u> Iron-regulated protein A putative	-	=
HD-9	86 % <i>V. cholerae</i> ChrI (113 of 251)	<u>VC1263</u> GTP cyclohydrolaseII	78 % <i>V. cholerae</i> ChrI (113 of 251)	<u>VC1263</u> GTP cyclohydrolase II

Table 5.7. Identity and closest gene matches from the BLAST database (EMBL) of the insert sequences from the genomic plasmid library haemolytic clones (HD-1 to HD-9). Sequences were obtained using M13 forward and reverse primers. ¹Indicates the section of the complete chromosome.

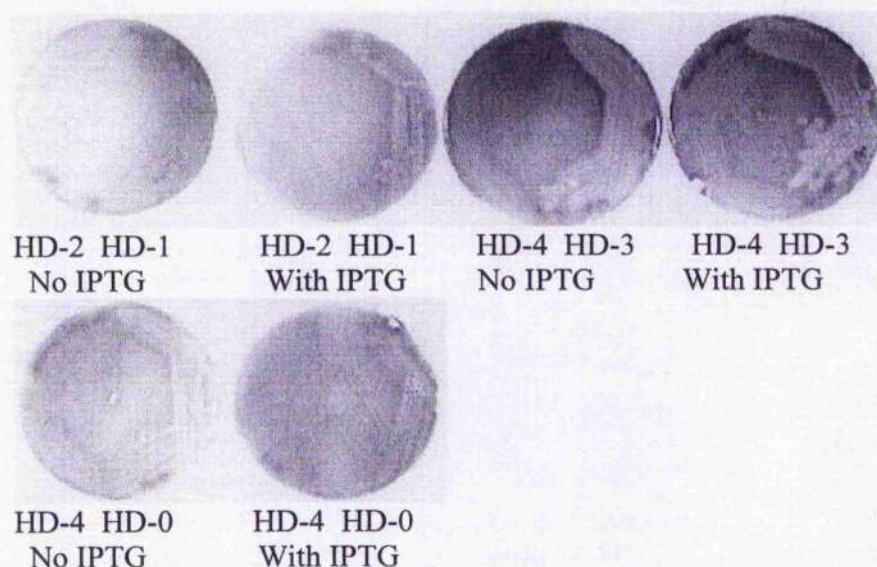


Figure 5.25. Effect of IPTG on haemolysis induced by clones (HD-1 to HD-4) from the *V. splendidus* DMC-1 genomic plasmid library on selective agar plates. HD-0 does not contain a DNA insert and was used as a negative control.

Clone	Haemolysis	
	IPTG added	No IPTG added
HD-1	++	+
HD-2	No growth	No growth
HD-3	++	++++
HD-4	+	+
HD-5	+	+
HD-6	-	-
HD-0 ¹	-	+

Table 5.8. Effects of IPTG on haemolytic activity expressed by selected clones (HD-1 to HD-6) from the *V. splendidus* DMC-1 genomic plasmid library. + = level of haemolysis; - = no haemolysis; ¹Non-haemolytic clone.

Another question was whether the orientation of the fragment within the plasmid vector had an effect on the expression of haemolytic activity. The plasmid vector pUC19 was used to orientate the HD-4 DNA fragment of 1.3 kb in the opposite direction to that in pUC18. These plasmid vectors are identical except that the sequences within the multiple cloning site are oppositely orientated. The DNA insert from HD-4 was digested out of the plasmid vector using restriction enzymes *EcoRI*

and *Hind*III and ligated into the plasmid vector pUC19 cut with the same restriction enzyme. These plasmids were expressed in *E. coli* TOP10 cells on selective blood agar plates to assess the effect on haemolytic activity. As shown in Figure 5.26 clones carrying the pUC19 vector with no insert caused haemolysis, an effect not seen with pUC18 alone. Therefore, the question of DNA insert orientation could not be answered with pUC19 due to the effects of the vector.

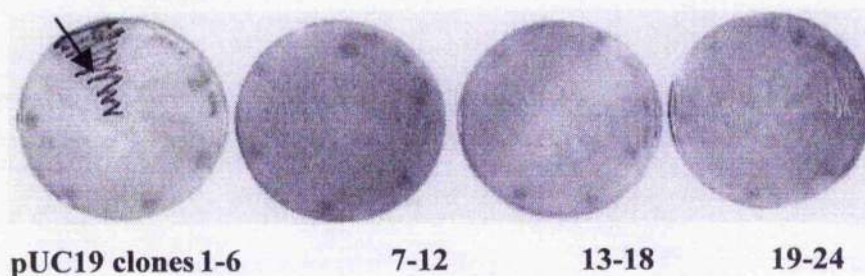


Figure 5.26. *Escherichia coli* TOP10 clones (1-24) containing plasmid vector pUC19 with the HD-4 DNA insert (1.3 kb). Clone 1 (indicated with arrow) does not contain a DNA insert and β -galactosidase activity is shown by reaction with the X-gal in the medium.

5.3.7.3. Lambda genomic library

Due to the difficulties experienced with the plasmid library, attention turned to production of a lambda library as this was capable of accepting larger DNA inserts, therefore allowing better coverage of the genomic DNA. The library was constructed in λ ZAP as described in section 5.2.11. The library preparation contained 7.8×10^6 pfu ml⁻¹ with an average insert size from single-clone excision experiment from 10 phage (Figure 5.27) of 4 kb.

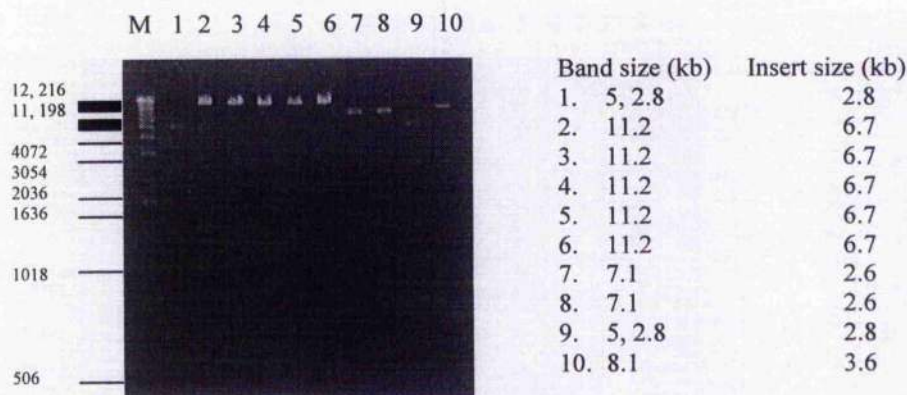


Figure 5.27. A selection of phagemids pBK-CMV containing lambda phage library DNA inserts digested with restriction enzyme *EcoRI*. The molecular weight marker used was 1 kb ladder (Promega), with sizes listed at the side of the gel. The total DNA insert size for each clone is listed alongside the band sizes.

The phage library stock was amplified, resulting in an increase from 7.8×10^6 pfu ml⁻¹ to 1×10^{10} pfu ml⁻¹. The original and amplified phage libraries were screened for haemolytic plaques, but none were found in the screening of approximately 10^5 plaques.

Therefore, due to the lack of success in identifying the haemolysin gene(s) using the two types of genomic DNA libraries an alternative approach of transposon mutagenesis was used.

5.3.7.5. Transposon mutagenesis

Initial work was carried out using the transposon Tn5 located within the plasmid vector pUT::miniTn5CmlacZ created by Dr. A. Hosie (PhD Thesis, 1998). Filter-mating was used to allow conjugation between donor and host strains, but no transposon exconjugants were produced despite varying the conditions for conjugation. A second attempt to use Tn5 was carried out using a pUT::miniTn5Cmlux vector. The protocol for mating was altered to incorporate the use of phage to kill the donor strain *E. coli* SM10λpir. Again, conjugation was unsuccessful and no mutants were produced. Despite the use of different protocols and vectors no

success was obtained with Tn5 and an alternative transposon (Tn10) was obtained from BioCentrum DTU, Denmark for further transposon mutagenesis work.

The Tn10 was located on the suicide vector plasmid pBSL181 carried in *E. coli* S17 λ pir cells. Tetracycline was the only selective antibiotic that could have been used for the combination of *E. coli* S17 λ pir and *V. splendidus*, but Hosie (1998) found that tetracycline could not be used in the presence of MgSO₄ in the mating plates as Mg²⁺ ions are antagonistic to this antibiotic. Therefore, the host strain was changed from *E. coli* S17 λ pir to *E. coli* Sm10 λ pir as this could be used with streptomycin and chloramphenicol to kill the host and recipient strains that did not contain the transposon insertion.

To optimise mutagenesis conditions with Tn10, a combination of changes to the protocol were explored. Alterations were made to the donor/recipient ratios, mating incubation times, wash solutions, growth medium, growth temperature for each strain, culture washes prior to mating and the antibiotic concentration used in the selective plates.

Various optimisation experiments were attempted to obtain a high number of exconjugants. Overall, the *E. coli* and *V. splendidus* strains grew optimally with agitation in LB at 37 °C and in MB at 20 °C, respectively (Figure 5.28). The poor growth of *V. splendidus* in LB broth was expected due to the lack of salt, as this is required for growth of this marine organism. To overcome this, the salt concentration in LB broth was altered and both donor and recipient growth rates were compared, (Figure 5.29). LB broth has a normal salt content of 1.0 %, which limits the growth of *V. splendidus* and when the salt concentration was increased, the growth of *V. splendidus* improved, as expected (Figure 5.29). The optimal combination of salt concentration for the two strains was 1.5 % (LB15). This concentration does not adversely affect the growth of the donor strain and allows the recipient strain to grow. This concentration of salt was applied to the mating plates to allow conjugation to proceed successfully by possibly favouring the donor and stressing the recipient strain to allow better uptake of the plasmid.

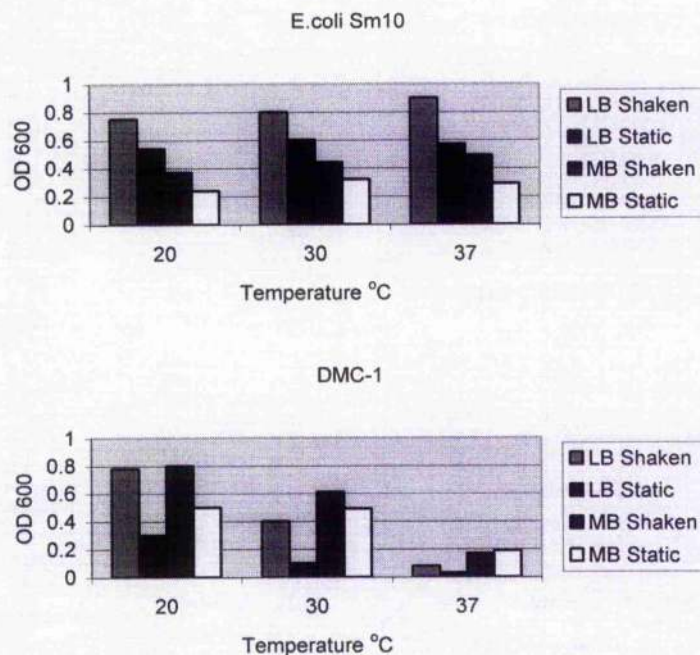


Figure 5.28. Temperature, growth medium and growth condition effects on donor (*E. coli* Sm10) and recipient strains (*V. splendidus* DMC-1) used in transposon mutagenesis experiments.

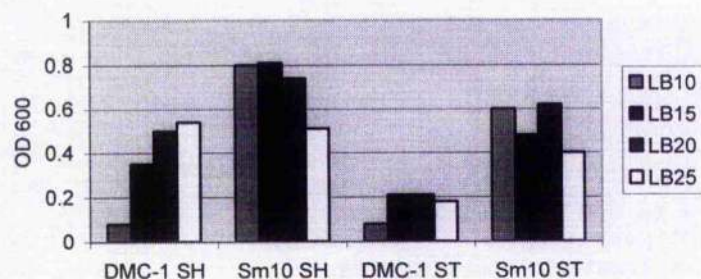


Figure 5.29. The effect of different salt concentrations on growth of transposon mutagenesis donor (*E. coli* Sm10) and recipient strains (*V. splendidus* DMC-1) at 37 °C and 20 °C, respectively. (SH = Shaken; ST = Static; LB10 = 1 % NaCl; LB15 = 1.5 % NaCl; LB20 = 2 % NaCl; LB25 = 2.5 % NaCl).

However, the different combinations of temperature and growth medium were no more beneficial in producing transposon mutants than using the optimal media and temperature suited for each strain. The main beneficial change to the protocol was in

the mating step where instead of using filter units, spots of cultures were placed directly onto the surface of agar plate surface as in the second Tn5 experiment.

A critical factor affecting the success of mutagenesis was the ratio of donor to recipient cells. As it was important to know the exact cell numbers for preparation of different ratios, numerous ratios were used at the mating stage. However, experiments that gave high numbers of exconjugants were at ratios of 1:1 and to keep as closely as possible to this ratio, a range of ratios was set up based on estimated cell numbers from optical density readings of the cultures followed by plating out to obtain true viable counts for each strain. An optical density of 1 for *E. coli* SM10 λ pir was equal to 3×10^9 cfu ml⁻¹ and for *V. splendidus* DMC-1 it was equal to 5×10^9 cfu ml⁻¹. Only when a 1:1 combination was achieved was the experiment continued and this resulted in successful production of numerous exconjugants. The number of mating spots was increased to scale up the numbers of exconjugants produced, enabling thousands of mutants to be screened for the lack of haemolytic activity.

During the optimisation stages any transposon mutants created were picked and stored at -80 °C for further screening. Initially they were screened for antibiotic resistance, haemolytic activity, oxidase activity and growth and activity on TCBS to confirm that the mutants were *V. splendidus*.

In total, over 10,000 mutants were created and screened for haemolytic activity resulting in three mutants that were haemolysin-negative and about 200 that were weakly haemolytic. Genomic DNA from the potential haemolysin-negative mutants was purified and used to carry out PCR with primers specific for the chloramphenicol resistance cassette located within the transposon. This confirmed the presence of Tn10 incorporated into genomic DNA (Figure 5.30). The three haemolysin-negative mutants formed from the mutagenesis work were designated DMC-1-M2, DMC-1-M3 and DMC-1-M4.

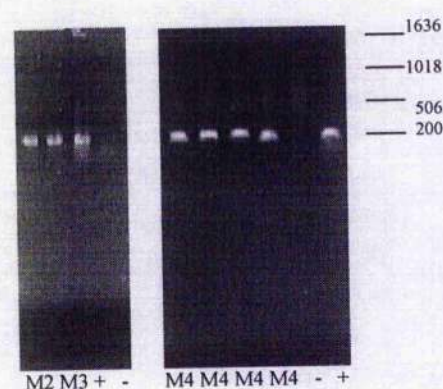


Figure 5.30. Analysis of the PCR products of the chromosomal DNA of the haemolysin-negative mutants *V. splendidus* DMC-1-M2, M3, M4. Product was amplified with primers designed against a 200 bp fragment of the chloramphenicol cassette (cat) located within the transposon Tn10. The 200 bp PCR product was present in all strains. The molecular weight marker used was 1 kb ladder (Promega) with the sizes (kb) listed at the side of the gel.

The haemolysin-negative mutants were all screened for haemolytic activity on blood agar plates and in the haemolysin assay (Figure 5.31-32). No difference was found in the protein concentration of the bacterial supernatants of these mutants compared with the wild-type DMC-1 and the mutant strains (Table 5.9) nor in the protein banding pattern for the DMC-1 wild-type strain and the DMC-1-M2 strain (Figure 5.33). Biochemical reactivities were also measured using API-ZYM (bioMérieux) test strips (Figure 5.34 and Table 5.10) and no differences were seen. The growth rate of the mutants was also compared to assess if the mutation resulting in lack of production of haemolysin had affected the growth rate. No significant difference was seen between the strains (Figure 5.35). Therefore, the lack of haemolysin was not as a result of differences in growth rate.

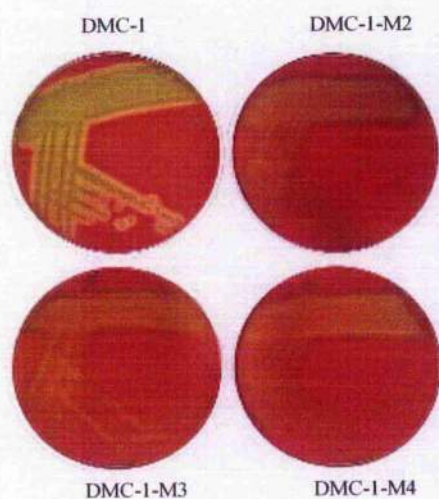


Figure 5.31. Haemolytic activity of *Vibrio splendidus* DMC-1 and mutant strains DMC-1-M2, M3, M4 on MA blood plates.

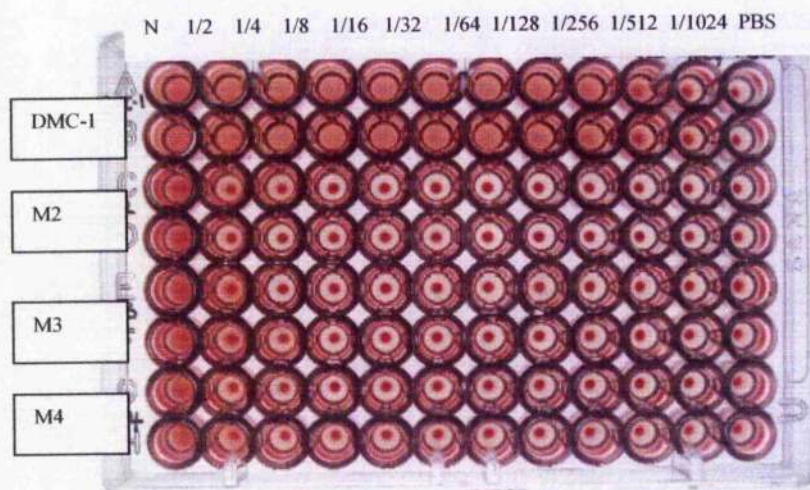


Figure 5.32. Haemolysin assay plate of *V. splendidus* DMC-1, DMC-1-M2, M3 and M4. Doubling dilutions of neat to 1/1024 were set up in duplicate for each strain. PBS was included as a negative control in the last column of the plate.

Strain	Haemolysin titre	Protein $\mu\text{g ml}^{-1}$
DMC-1	1/256	58.1
DMC-1-M2	- ¹	58.3
DMC-1-M3	-	65.6
DMC-1-M4	-	63.4

Table 5.9 . Haemolytic activity and protein concentration in bacterial culture supernatants of *V. splendidus* and haemolysin negative mutants DMC-1-M2, M3, M4.

¹no lysis detected with neat samples.

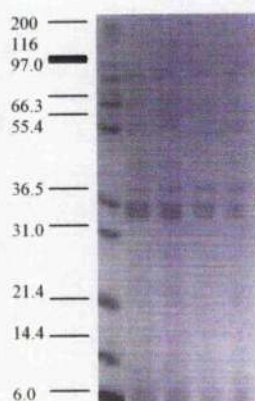


Figure 5.33. Analysis of bacterial culture supernatants of the wild-type *V. splendidus* DMC-1 and DMC-1-M2 on SDS-PAGE gel. Mark12 marker was used as the standard protein marker, with protein sizes (kD) listed at the side of the gel.

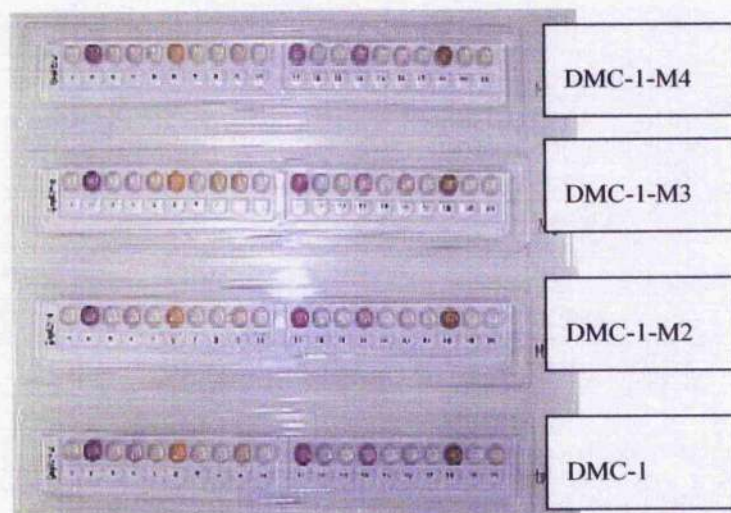


Figure 5.34. API-ZYM test strips of bacterial culture supernatants of *V. splendidus* DMC-1 and haemolysin negative mutant strains *V. splendidus* DMC-1-M2, M3, M4.

API-ZYM	DMC-1	DMC-1-M2	DMC-1-M3	DMC-1-M4
Alkaline phosphatase	1	1	1	1
Esterase (C4)	1	1	1	1
Esterase Lipase (C8)	1	1	1	1
Lipase (C14)	0	0	0	0
Leucine arylamidase	1	1	1	1
Valine arylamidase	0	0	0	0
Cystine arylamidase	0	0	0	0
Trypsin	1	1	1	1
α -chymotrypsin	0	0	0	0
Acid phosphatase	1	1	1	1
Naphthol-AS-BI-phosphohydrolase	1	1	1	1
α -galactosidase	0	0	0	0
β -galactosidase	1	1	1	1
β -glucuronidase	0	0	0	0
α -glucosidase	1	1	1	1
β -glucosidase	0	0	0	0
N-acetyl- β -glucosaminidase	1	1	1	1
α -mannosidase	0	0	0	0
α -fucosidase	0	0	0	0

Table 5.10. API-ZYM results for wild-type and transposon mutant *V. splendidus* DMC-1 strains. 1 = Positive, 0 = Negative.

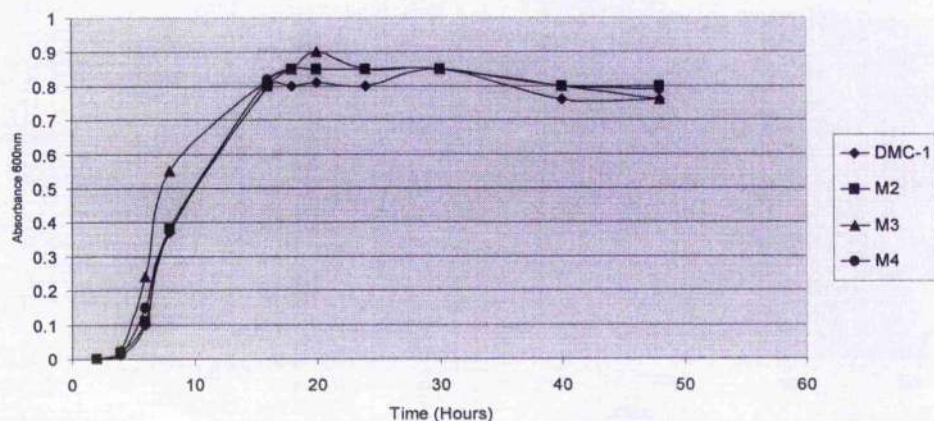


Figure 5.35. Growth curves of the *V. splendidus* DMC-1 parent and mutant strains DMC-1-M2, M3 and M4. Optical density of bacterial cells was measured at 600 nm over 48 h.

Identification of the gene into which the inserted genetic element had transposed required genomic DNA for molecular analysis and this was purified and digested with the restriction enzyme *Sph*I to obtain fragments containing the intact transposon. The fragments were ligated into the plasmid vector pUC18, which was heat shock transformed into *E. coli* TOP10 cells and the transformants selected on LB selective plates containing chloramphenicol and ampicillin, such that only clones containing the plasmid with the DNA insert containing transposon Tn10 were selected.

From the resulting clones, plasmids were purified from the bacteria and the insert excised by digestion with *Eco*RI. The sizes of the fragments for each of the haemolysin-negative mutants were: DMC-1-M2, 4.5 kb; DMC-1-M3, 4.5 kb and DMC-1-M4, 3.5 kb. The plasmids containing these inserts were initially sequenced with M13 forward and reverse primers revealing that transposon mutants DMC-1-M2 and DMC-1-M3 were identical (Appendix 3); and that these mutants had probably originated from the same mutagenesis event. The DMC-1-M2 *Sph*I fragment was fully sequenced and the position of the transposon was located as being in a gene with homology to a hypothetical protein close to a sodium/alanine symporter from *Vibrio* spp.

The sequences for DMC-1-M4 with M13 forward and reverse primers are also given in Appendix 3. Here, the position of the transposon insertion was defined as being in a gene with homology to the aerolysin gene from various *Aeromonas* spp. Further sequencing and characterisation of the gene(s) surrounding the transposon insertions in DMC-1-M2 and DMC-1-M4 are covered fully in chapter 6.

5.3.8. Challenge trials with haemolysin-negative mutants

5.3.8.1. *In vitro* trials

5.3.8.1.1 Haemolysin assay

As already discussed all the mutants lacked haemolytic activity against sheep red blood cells (Figure 5.32 and 5.36).

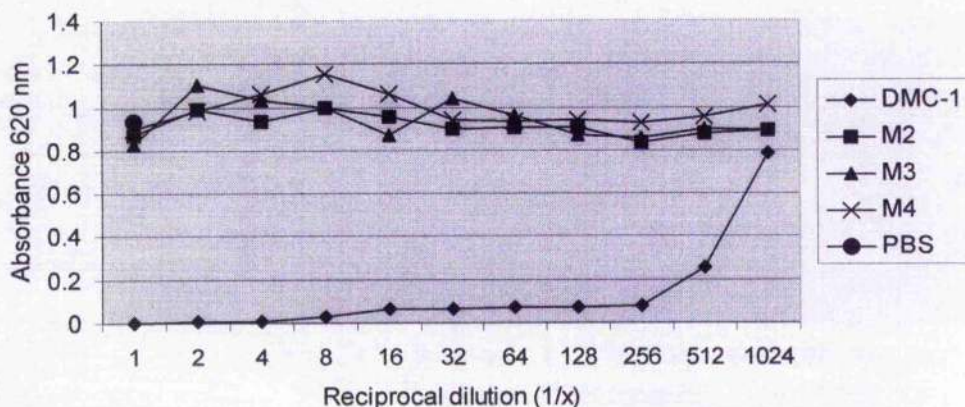


Figure 5.36. Quantification of haemolysin in bacterial culture supernatants of *V. splendidus* DMC-1 and the mutant strains *V. splendidus* DMC-1-M2, M3 and M4. Doubling dilutions of filter-sterilised bacterial supernatants were set up to titrate the haemolytic activity with PBS as a negative control. Haemolytic activity was measured using cell scattering data at an absorbance of 620 nm.

5.3.8.1.2 Activity against tissue culture cells

To assess the cytotoxic activity of the mutants, TV1-S4 cells were exposed to bacterial cells and supernatant. Unfortunately, the salt conditions were too low for the bacterial cells to remain viable, even after gradual reduction of the salt levels in the wash buffer. However, the filter-sterilised culture supernatants of the DMC-1 parent strain caused degenerative changes to the monolayer, which consisted mainly of the development of clusters of round cells, dendritic elongations and finally complete detachment of the cells after overnight incubation. In contrast the culture supernatants from the mutants showed no visible effect on the cell monolayer that remained exactly as in the control wells. Therefore, these mutants had lost their cytotoxic activity to TV1-S4 cells as well as haemolytic activity that suggests the haemolysin is responsible for the damaging effects on both these cells types.

5.3.8.2. *In vivo* trials

5.3.8.2.1 Turbot larval trials in Bergen

To test the effects of the mutant strains *in vivo*, infection trials with turbot larvae were carried out at two different stages of growth, the yolk-sac phase and first-feeding

larvae. The data for the DMC-1 parent strain has previously been shown in chapter 3 for all trials.

The two yolk-sac trials included the mutant strains, DMC-1-M2 and DMC-1-M3 in addition to the DMC-1 strain. As discussed in chapter 3 the DMC-1 strain had no effect on yolk-sac larvae with very few mortalities, and similar results were obtained with the mutant strains (Figure 5.37).

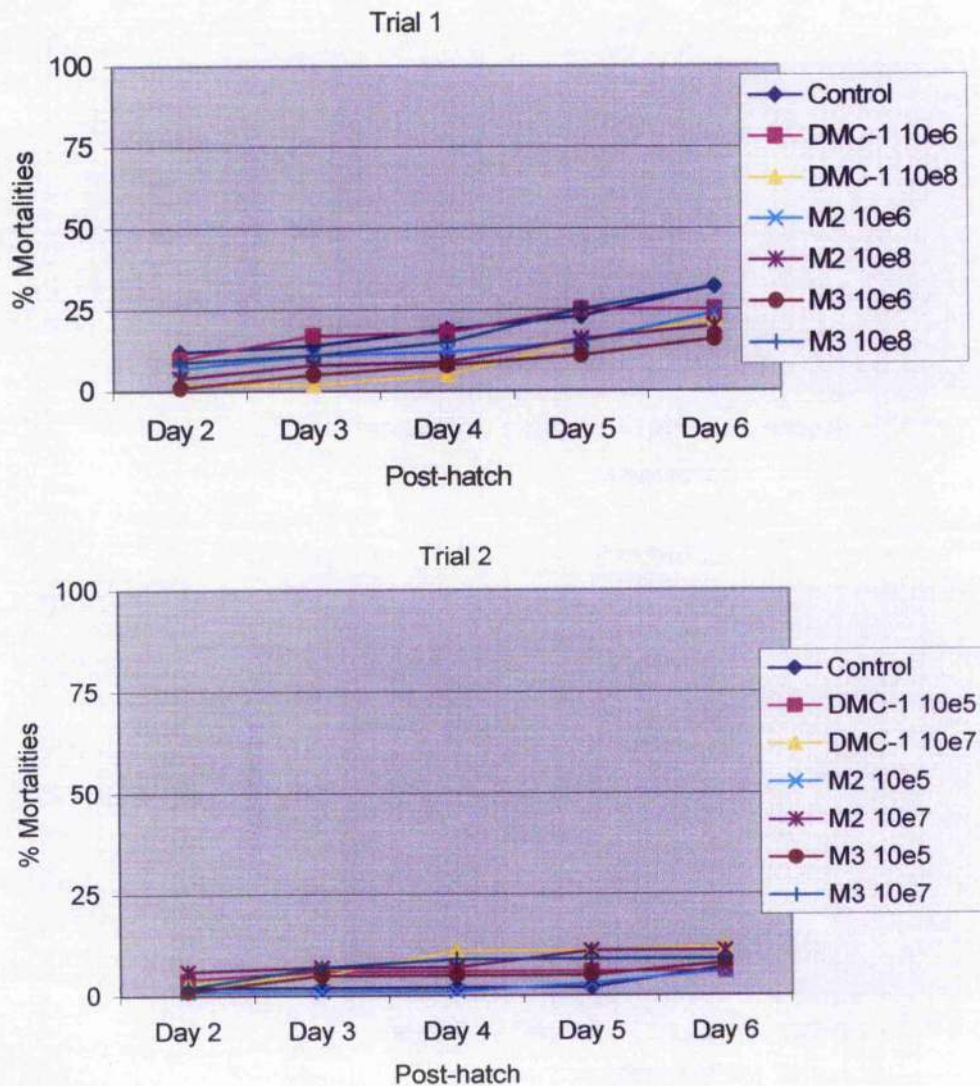


Figure 5.37. Turbot yolk-sac larval trials with wild-type *V. splendidus* DMC-1 and haemolysin negative mutants *V. splendidus* DMC-1-M2 and *V. splendidus* DMC-1-M3. Two separate trials were conducted and the concentration of bacteria to which larvae were exposed is shown in the legends.

With the first-feeding trials, two experiments were carried out, one with DMC-1-M2 and one with DMC-1-M4, respectively. The protocols were different for each trial as described earlier (section 5.2.16). The first trial, with strain DMC-1-M2 showed that the parent strain DMC-1 gave significantly higher mortalities than the control and DMC-1-M2 groups, with DMC-1-M2 behaving in an identical manner to the control group, (Figure 5.38). The immunohistochemistry results showed that DMC-1-M2 colonised the gut but did not damage or invade the gut cells (Figure 5.39, A1-2) to cause cellular destruction as was seen in the DMC-1 group (Figure 5.39, B2, arrow 5). The immunohistochemistry results for DMC-1 were covered in Chapter 3, section 3.3.5.1. Only one control group was included in this study due to a crash in the other control tank on the second day of the trial. The increase in mortalities at the end of the trial is as normally experienced in the Bergen trials unit.

Overall, this trial showed the pathogenic activity of the parent strain in comparison to the non-pathogenic haemolysin-negative mutants and that knocking out the haemolytic activity resulted in reduced virulence towards first-feeding turbot larvae.

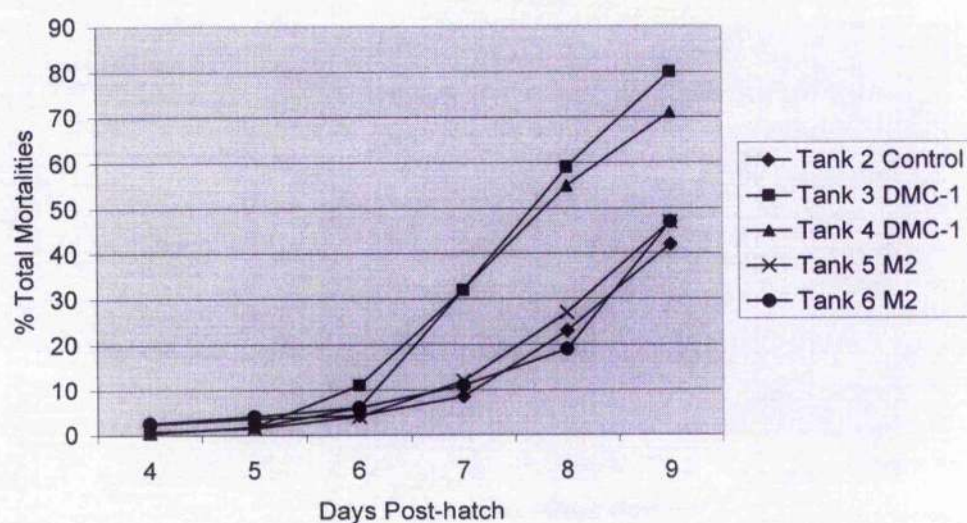


Figure 5.38. Turbot larvae first-feeding trials with wild-type *V. splendidus* DMC-1 and haemolysin negative mutant *V. splendidus* DMC-1-M2. Turbot larvae were challenged on days 4 and 5, with challenge bacteria added to the live food rotifers to give a final bacterial concentration of 3×10^4 cfu ml⁻¹ in each challenge tank. This trial was carried out in the challenge facilities in IMR, Bergen.

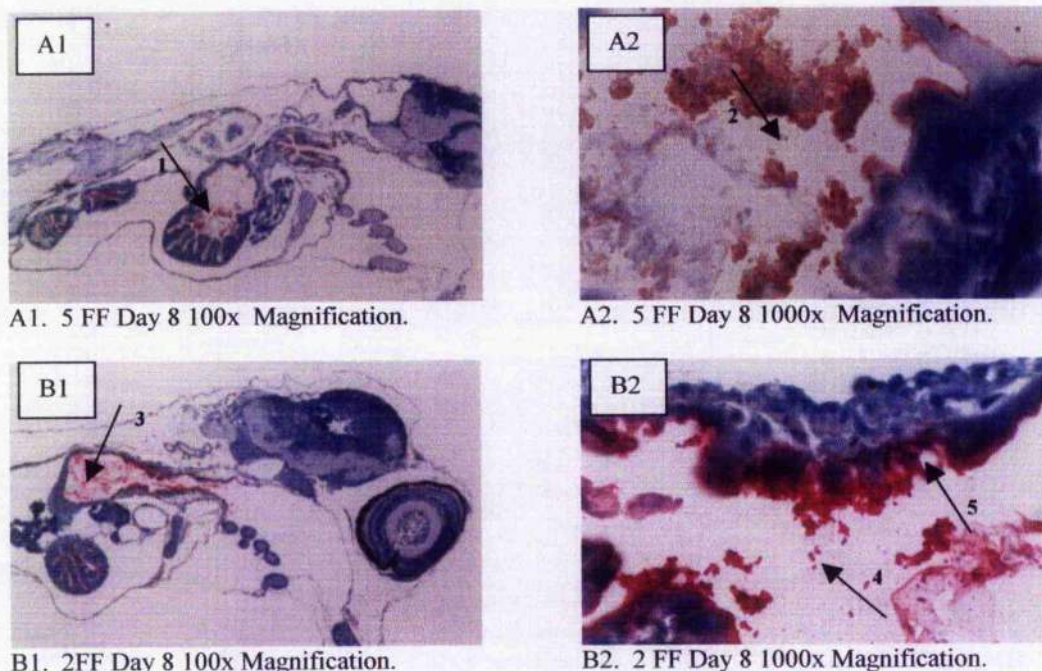


Figure 5.39. Immunohistochemistry slides of larval turbot sections from first-feeding trial at IMR, Bergen. A1 and A2, *V. splendidus* DMC-1-M2 (5 FF = Group 5 of first-feeding trial); B1 and B2, *V. splendidus* DMC-1 (2 FF = Group 2 of first-feeding trial) challenged first-feeding (FF) larvae sampled on day 8. **Arrows 1 and 3** = DMC-1 and DMC-1-M2 stained cells in the larval gut, respectively; **2 and 4** = Individual bacterial cells, **5** = Damage to the brush border.

5.3.8.2.2 Turbot larval trial-Glasgow

The second trial was carried out in Glasgow with the mutant strain DMC-1-M4, and a different protocol was followed. A smaller scale challenge was set up for 6 days with the larvae receiving DMC-1-M4 daily from day one and challenge with the pathogen DMC-1 on three consecutive days. Due to the lack of haemolysin production and the avirulence of M2 *in vivo* (section 5.3.8.2.1), a challenge was carried out to assess whether the other mutant, DMC-1-M4 strain could protect against infection with *V. splendidus* DMC-1. The results in Figure 5.40 show that the mortalities in the control group reached 55 % on day 10 post-hatch. Chi-square tests were used to compare the percentage of mortalities between the groups (Table 5.11) and from the p-values for day 9 there was a significant difference between the control group and DMC-1, with higher mortalities in the latter. However, this was not the case on day 10 when the p-

value increased to > 0.05 . The p-value for DMC-1 versus DMC-1-M4 is 0.06 on day 9, which means there is no significance between them. On day 10 there was a significant difference with a p-value of 0.02 but there were no significant differences between the control and DMC-1-M4 groups on either day 9 or 10. Overall, the control group mortalities were higher than expected, and limited conclusions can be drawn in this experiment.

The bacteriology of the larval gut and the tank water was measured on day 9 to assess the levels of *V. splendidus* DMC-1 versus other bacteria, (Figure 5.41). *Vibrio splendidus* DMC-1 and DMC-1-M4 were detected using agglutination assays with DMC-1 specific antisera. It was found that DMC-1 dominated the gut flora of the DMC-1 group, the same effect was shown with DMC-1-M4 strain, with no detection of DMC-1. Therefore, the DMC-1-M4 strain has not lost the ability to colonise the gut and thus may be a suitable competitor to prevent DMC-1 infection. A similar pattern was seen in the tank water samples. Therefore, if the mutant strain is non-pathogenic and colonises the gut it could be used as a potential probiotic against *V. splendidus*.

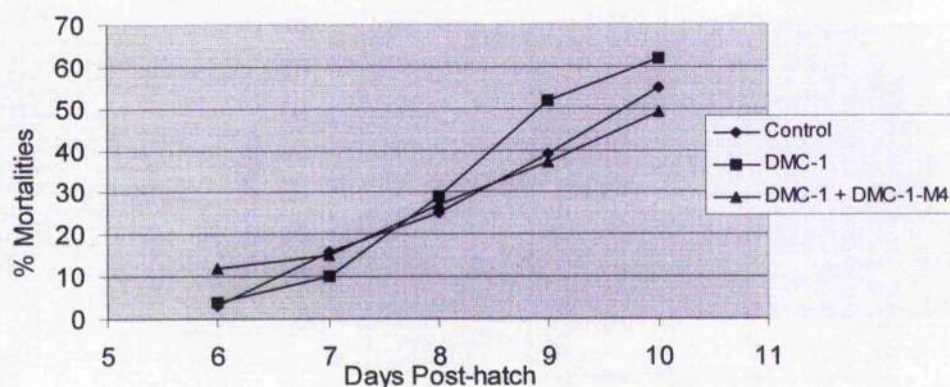


Figure 5.40. Mortalities in first-feeding turbot larvae exposed to wild-type *V. splendidus* DMC-1 alone and together with the haemolysin negative mutant *V. splendidus* DMC-1-M4. Turbot larvae were challenged with *V. splendidus* DMC-1 on days 5-7 with the live food rotifers at a concentration of 1×10^7 cfu ml⁻¹ (final concentration in tank was 3×10^4 cfu ml⁻¹). The second challenge group received *V. splendidus* DMC-1 in the same manner, with these tanks also having an addition of *V. splendidus* DMC-1-M4 from days 2-10 post-hatch along with the rotifers (1×10^7 cfu ml⁻¹, final concentration 3×10^4 cfu ml⁻¹). Challenge trial was carried out in the Division of Infection and Immunity, University of Glasgow.

Groups	p-value		Significantly different	
	Day 9	Day 10	Day 9	Day 10
Control vs. DMC-1	0.028	0.241	Yes	No
Control vs. DMC-1 + DMC-1-M4	0.722	0.248	No	No
DMC-1 vs. DMC-1+ DMC-1-M4	0.062	0.020	No	Yes

Table 5.11. Chi-square test values for larval mortalities in challenge groups of *V. splendidus* DMC-1 alone and in a joint challenge with the transposon mutant *V. splendidus* DMC-1-M4 on day 9 and 10 of the Glasgow first-feeding trial.

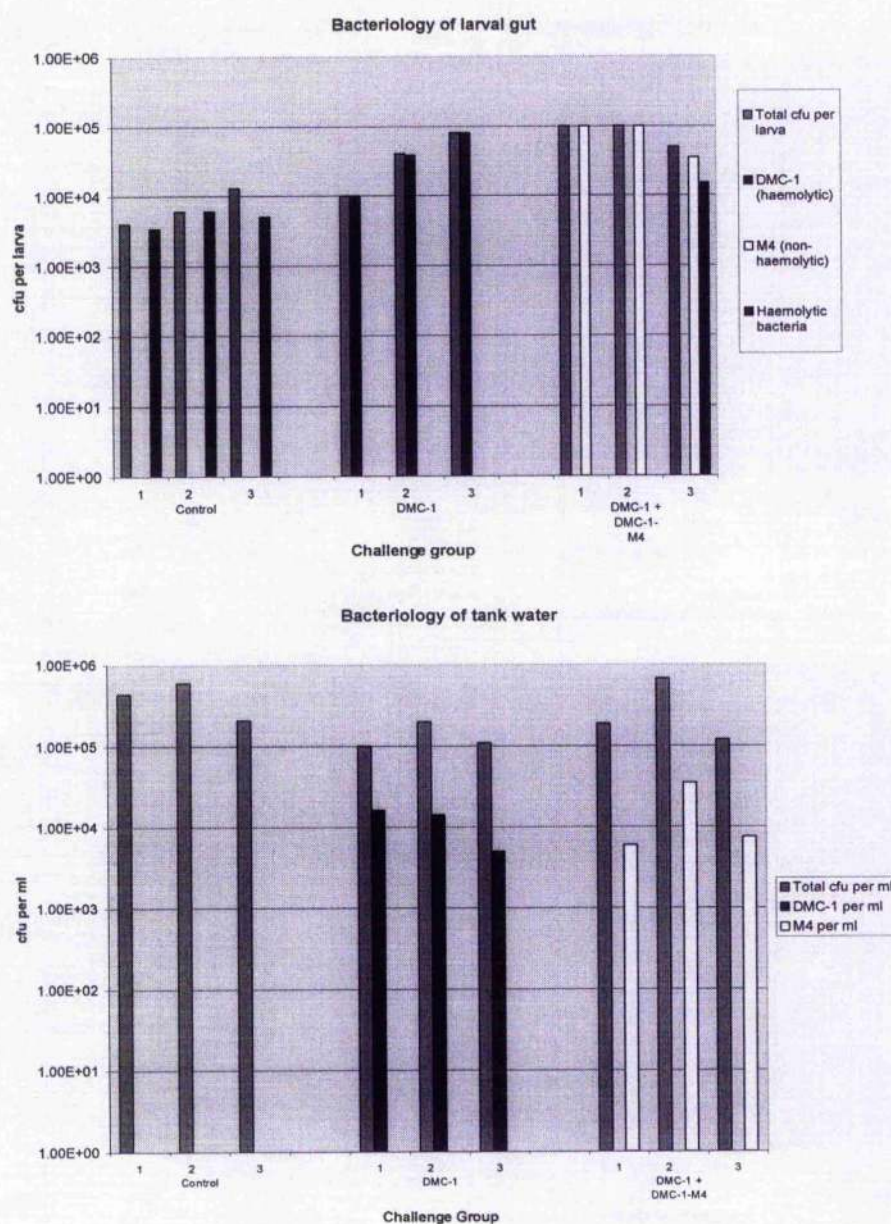


Figure 5.41. Concentration of bacteria in the larval gut and tank water for unchallenged turbot larvae (control, tanks 1-3), larvae exposed to *V. splendidus* DMC-1 alone (DMC-1, tanks 1-3) and larvae exposed to both *V. splendidus* DMC-1 and *V. splendidus* DMC-1-M4 (DMC-1 + DMC-1-M4, tanks 1-3) on day 9. Five larvae from each individual tank (1-3) for each challenge group were pooled together, surface sterilised and homogenised to obtain bacterial counts for each tank in each challenge group. Tank water was sampled from each tank in each challenge group. Haemolytic bacteria were detected by culture on marine blood agar and the identity of isolates believed to be *V. splendidus* DMC-1 or *V. splendidus* DMC-1-M4 were confirmed in agglutination assays with antisera to *V. splendidus* DMC-1.

5.4. Discussion

The haemolysin produced by *V. splendidus* DMC-1 is heat-labile, optimally produced during late exponential growth phase (between 16-24 h) when a high titre of the toxin was detected. The levels of toxin fell after this phase of growth perhaps as a result of loss of cell viability or perhaps due to proteolytic degradation of the toxin. If the latter occurs, one could add protease inhibitors during the exponential phase of growth that could result in increased haemolysin yields. Alternatively, the toxin may be highly reactive and oligomerise or bind to surfaces.

The toxin action was also studied in this chapter, and preliminary work was carried out assessing the affect of temperature on haemolytic activity. The haemolysin bound to erythrocytes in a temperature-independent manner, whereas haemolysis occurred in a temperature-dependent manner. This effect has also been seen with *V. mimicus* haemolysin (Shinoda *et al.*, 1993), *V. vulnificus* cytotoxin (Gray and Kreger, 1985) and *V. parahaemolyticus* thermolysin, TDH (Honda *et al.*, 1992, Douet *et al.*, 1996). However, the opposite was reported for the cytotoxin produced by *V. metschnikovii* (Miyake *et al.*, 1989).

The haemolysin was identified as a pore-forming toxin and toxins with similar modes of action have been identified from many pathogenic vibrios, including the El Tor haemolysin, ETH (HlyA) of *V. cholerae* (Ikigai *et al.*, 1996, Huntley *et al.*, 1997), TDH of *V. parahaemolyticus* (Honda *et al.*, 1992) and *V. mimicus* haemolysin, VMH (Shinoda *et al.*, 1993) using colloid-osmotic lysis experiments. In other bacterial species, important pore-forming toxins include the α -toxin of *Staphylococcus aureus*, *E. coli* haemolysin (Bhakdi *et al.*, 1996) and aerolysin from *Aeromonas* species (Garland and Buckley, 1988; Fivaz *et al.*, 2001).

In general pore-forming toxins attack cells by forming oligomers on the cell surface, thereby creating channels in the infected cell membranes. These channels allow water to permeate which results in a physical destruction of the cell by increasing the intracellular colloidal osmotic pressure. Most pore-forming toxins have been identified as being haemolysins, however their action on other cells *in vivo* may just be as important if not more. The cytotoxic action of the *V. splendidus* haemolysin on

other cells types was identified in the turbot tissue culture TV1-S4 assay as well as in the *in vivo* trials with first-feeding larvae where damaging effects on the larval gut cells were observed. Using the osmotic protection experiments with a series of polyethylene glycols, the size of the pore was estimated to be between 1.8-1.9 nm in diameter. Although this does not provide the most accurate measurement of actual pore size it does allow comparisons to be made with toxins tested in similar experiments. For example, the pore-size of the El Tor haemolysin, ETH of *V. cholerae* is between 1.2 and 1.6 nm (Ikigai *et al.*, 1996), TDH from *V. parahaemolyticus* is between 2 and 3 nm (Honda *et al.*, 1992), and *E. coli* haemolysin is between 2 and 3 nm (Bhakdi *et al.*, 1986). However, larger pore-sizes have been recorded from the *V. mimicus* haemolysin at 2.8-3.5 nm (Shinoda *et al.*, 1993) and aerolysin at 3 nm (Howard and Buckley, 1982). Overall, the pore-forming toxins are very important virulence factors as shown from the damaging effects of vibrio haemolysins, aerolysin and α -toxin of *Staphylococcus aureus* (Braun and Focareta, 1991), emphasising the importance of this haemolysin as a major virulence factor.

Other studies carried out with the toxin included assessing the effects of metal ions on haemolytic activity. Initial results showed that $ZnSO_4$ and $MnSO_4$ inhibited haemolysis but later the results were inconclusive for $MnSO_4$, and only $ZnSO_4$ inhibited the activity at concentration between 1 and 5 mM. Harshman and Sugg (1985) studied the effect of calcium and zinc ions on the action of *S. aureus* α -toxin. Both these ions inhibited the action of this pore-forming toxin and calcium impeded the formation of pores by decreasing the mobility of oligomer complexes after the toxin had bound to the membrane.

Haemolysins produced by other vibrios have also been shown to be inhibited by divalent cations, including VMH of *V. mimicus* (Shinoda *et al.*, 1993 and Miyoshi *et al.*, 1997), *V. cholerae* El Tor haemolysin (Zitzer *et al.*, 1995), haemolysins of *V. vulnificus* (Shinoda *et al.*, 1985) and *V. metschnikovii* (Miyake *et al.*, 1989). The mechanism by which these inhibitory cations act is thought to be by a reversible action on the lipid bilayer that makes the membrane rigid or decreases fluidity (Miyake *et al.*, 1989). As a result, the cytolysin is unable to move in the membrane to form pores, as described for *S. aureus* α -toxin (Harshman and Sugg, 1985). It has also been suggested that this inhibition is due to an osmotic protection mechanism

that blocks the influx of water such that no haemolysis occurs (Miyoshi *et al.*, 1997). The ZnSO_4 may inhibit haemolytic activity in either of these ways and further experiments are necessary to confirm the mode of inhibitory action.

The purification of many extracellular haemolysins and proteins has involved the use of ammonium sulphate precipitation and column chromatography. However, as was discussed in chapter 4, ammonium sulphate precipitation was not an ideal method as the activity of the haemolysin became unstable. Therefore, an alternative method using ultrafiltration with an Omega filtron system was employed to concentrate a large volume of bacterial supernatant. Overall, this method of concentration allowed production of crude toxin with a high haemolytic activity that remained stable, and this material was used throughout the column chromatography purification studies as the starting active concentrate.

Unfortunately, the protein purification techniques used were not successful in yielding workable quantities of purified toxin. Ion-exchange chromatography was the most successful in producing partially-purified toxin, but this only increased the specific activity 5-fold. The hydrophobic interaction column was unsuccessful with only a 1-2 fold increase in specific activity from the concentrated material. In the purification of the *V. mimicus* haemolysin the specific activity was increased 500-fold (Miyoshi *et al.*, 1997) and in *Aeromonas salmonicida* the salmolysin specific activity was increased 945-fold (Nomura *et al.*, 1988) following column chromatography purification. However, the latter results are not convincing. Overall, the levels of toxin obtained from the purification of *V. splendidus* haemolysin were very low.

The active fractions from the ion-exchange column were analysed individually on SDS-PAGE and showed bands present at 35 and 29 kD. When these active fractions were pooled together and analysed again on SDS-PAGE, a large band was visible at >200 kD. This large band was not dominant in the concentrated supernatant and it is possible that when the fractions were pooled together and concentrated in the Diaflo membrane system, the haemolysin oligomerised into aggregates. On heat-treatment at 70 °C and 100 °C there was partial dissociation of this larger band to produce

additional bands at 53 kD and 29 kD, thus, providing evidence that this larger protein band may be an aggregate of the monomeric toxin.

Similar aggregation was seen with the El Tor haemolysin of *V. cholerae* O1 (Ikigai *et al.*, 1996). In which two haemolytic proteins of 350 kD and 65 kD were purified from the supernatant using Sephadex G-100 column chromatography. On heat-treatment the larger of the two dissociated to produce more of the 65 kD band. Further work with the ETH, using phosphatidylcholine-cholesterol liposomes and erythrocyte ghost membranes confirmed the existence of these oligomeric aggregates with sizes ranging from 170 to 350 kD. The size of the oligomers cannot be accurately estimated by gel electrophoresis as one cannot determine the precise number of subunits.

Smaller bands present in the haemolytic fractions may be a result of cleavage of the toxin. There is evidence that some toxins are cleaved in the extracellular environment to produce an active form of toxin. Again, this was the case for the ETH which produced two band sizes (55 and 65 kD) during immunoblotting experiments and both reacted with ETH antiserum (Ikigai *et al.*, 1996). Therefore, other bands present in the active fractions from column purification work may be different forms of the same toxin.

To identify whether this was the case, native gels were used to analyse which bands were associated with haemolytic activity. As shown in Figure 5.13 there was only 1 band corresponding to haemolytic activity. Ideally the dominant band present in this native gel that gave this corresponding haemolytic zone in the blood overlay gel would have been electro-transferred to PVDF membrane to enable N-terminal sequencing to be performed. However, it was thought necessary to remove further contaminating proteins from the fractions before this was attempted.

The hydrophobic interaction chromatography method used did not increase specific activity to adequate levels and was discarded. However, it was useful in the analysis of the protein bands on SDS-PAGE and in the native gel for comparison with the ion-exchange fractions. With the SDS-PAGE gel there was no large band at >200 kD, only a band at 50 kD. On the native gel no activity was produced in the blood overlay but the corresponding stained gel did show a dominant band in the same position as

that present in the native PAGE gel of the Q-column fractions (Figure 5.15). The lower level of haemolytic activity in these fractions may explain why no reaction was produced in the blood overlay of the native gel. The lower activity may be due to the loss of co-factors that had become bound to the column matrix. Proteases may also be present in the concentrated fractions alongside the haemolysin and be capable of degrading the toxin and causing a reduction in activity. Ideally, protease inhibitors should have been included in the buffer solutions to prevent any such degradation.

The Q-column fractions were also applied directly to a gel filtration column and SDS-PAGE bands of >200 kD were purified (with associated haemolytic activity) alongside bands of 40, 52 and 53 kD. The larger band, as mentioned previously, may be an active aggregate of this pore-forming toxin.

Overall, purification of the haemolysin using column chromatography proved difficult with no conclusive results being obtained. An additional method of purification was IEF, but again this was unsuccessful in retaining the activity of the toxin. Separation of proteins from the concentrated supernatant to their pI points caused an overall loss of haemolytic activity but indicated that the pI for this toxin was between 6.5-6.9. However, this was based on the fractions with the highest haemolytic activity and did not take account of other fractions that had lower haemolytic activity. Therefore, IEF produced similar results to chromatography with no purified toxin being obtained.

The overall conclusion was that the toxin was not purified as it became unstable and possibly degraded. The HlyA haemolysin of *V. cholerae* initially proved difficult to purify to a homogeneous toxin preparation without degradation using similar protein purification methods, (Hall and Drasar, 1990, Yamamoto and Kaper, 1990, Braun and Focareta, 1991). As an alternative to these protein purification methods, recombinant DNA techniques were employed, as is the case for many bacterial toxin studies where toxins have proved difficult to purify. By using such studies the toxins can be identified through sequences allowing the protein size, amino acid sequence and structure to be deduced.

The common technique of creating genomic libraries has been used in haemolysin (Jores *et al.*, 2003) and protease (Cascon *et al.*, 2000) characterisation studies. In this

case the creation of both a plasmid and lambda genomic library allowed the genomic DNA of *V. splendidus* DMC-1 to be analysed for the haemolysin gene(s). From analysis of both genomic libraries, four haemolytic clones were identified from the pUC18 library but the DNA sequences of these haemolytic clones did not match any specific haemolysin genes. Table 5.7 shows the matches against sequences in a BLAST database search and although the sequences matched genes from vibrio species none were obviously related to haemolytic activity or appeared likely to be relevant. As a result of these analyses it was questioned whether these clones carried specific haemolysin genes. The use of pUC19 to reverse the orientation of the insert proved to be unsuccessful as the pUC19 vector alone in the *E. coli* TOP10 cells produced a haemolytic effect, making it impossible to assess the haemolytic effect of the relevant inserts. In conclusion, it was decided that these clones did not contain the DNA of the haemolysin and transposon mutagenesis was then investigated.

Transposon mutagenesis is normally employed to identify specific virulence genes, whereby the transposable element inserts at random into DNA sequences thus disrupting one or more genes. In this case it was employed to mutate the haemolysin gene(s) to enable identification and as this trait could be assessed on blood plates it was easy to screen a large number of mutants.

Transposon mutagenesis has been applied to many characterisation studies of virulence factors. For example insertional mutagenesis has been used in *Vibrio* species to identify the extracellular cytotoxin of *V. vulnificus* (Wright and Morris, 1991), the major surface flagellar sheath antigen of *V. anguillarum* (Norqvist and Wolf-Watz, 1993), the flagellar filament of *V. anguillarum* (O'Toole *et al.*, 1996) and in another bacterial genus, the bacilysin of *Bacillus subtilis* (Yazgan *et al.*, 2001).

The transposons used in this study were located on suicide plasmid vectors, vectors that replicate in the donor strain but fail to do so in the recipient strain. After conjugation of the plasmid to the recipient strain the transposon uses a transposase located on the plasmid to move into the genomic DNA at random. Therefore, a range of mutations is created at random throughout the genome. As a result the mutants can be screened for lack of certain virulence factors, in this case the lack of haemolytic activity. The successful mutants created can also be used to assess the importance of

the virulence factor as was seen in transposon mutagenesis of *V. vulnificus* genomic DNA resulting in deletion of the cytolysin gene. However, when the mutant strain was applied to a mouse model reduced virulence was not found (Wright and Morris, 1991).

Transposon insertion into the target gene allowed the location of the mutation to be precisely mapped resulting in the identification of the gene(s). It is widely known that transposon mutagenesis with *Vibrio* spp. can prove difficult. As was shown in the results section numerous conditions were optimised in an attempt to achieve a high number of mutants to enable the genome to be covered. Mutagenesis with the transposon Tn5 proved unsuccessful but fortunately Tn10 proved successful. It was found that for mutagenesis to be successful in this strain it was important to use a donor to recipient ratio as close to 1:1 as possible before successful conjugation was achieved. Following the protocol of Solano *et al.* (2000) the mating technique was also altered from filter-mating to spotting of each culture onto the surface of an agar plate. From the large bank of transposon mutants produced, three were haemolysin negative (approximately 1 per 3500 mutants).

Initial screening of transposon mutants revealed that the DMC-1-M2 mutant had an interruption in a gene that did not match any specific haemolysin genes of any bacterial species. Nevertheless, the haemolytic activity was impaired and the mutant was found to have lost cytotoxic activity. The second mutant DMC-1-M4 was then identified as containing an interruption in a gene that had homology with aerolysin genes from several *Aeromonas* species that are in fact members of the Vibrionaceae family. This strain had also lost cytotoxic activity.

As a result, further sequencing was carried out on the DMC-1-M4 strain to identify the full haemolysin gene as well as genes up and down-stream of it. From this, the relationship between DMC-1-M2 and DMC-1-M4 was determined from further sequencing as discussed in chapter 6.

Chapter 6. Determination of the gene sequence for the *Vibrio splendidus* haemolysin.

6.1. Introduction

As discussed in previous chapters haemolysins are important virulence factors in many pathogenic strains of *Vibrio* spp. This is the first work known to be carried out on the characterisation of a haemolysin from a *V. splendidus* strain, and by transposon mutagenesis, two different haemolysin-negative mutants were created. Chapter 5 described the initial stages of identification of the genes into which the transposon, Tn10 had inserted. The virulence of mutants was shown to be attenuated in *in vivo* turbot larval challenge trials. This chapter is concerned with DNA sequence analysis in the region of the Tn10 insertion sites of the mutants DMC-1-M2 and DMC-1-M4 to characterise the haemolysin gene and its location within the *V. splendidus* genome.

6.2. Materials and Methods

6.2.1. Sequencing

Sequencing reactions were carried out at the DBS Genomics Unit at Durham University on an Applied Biosystems 377 DNA Sequencer XL. A 15 µl volume of DNA plasmid preparation was required for each sequencing reaction. The results were delivered via email and analysed on a Viglen Contender C466 computer with the GeneBuilder Program.

6.2.2. Primer design

Primers were designed for all sequencing reactions using the Primer3 program on the UK HGMP website. A list of primers used to sequence the haemolysin gene and areas up and downstream of the gene is shown in Appendix 2.

6.2.3. Sequence alignments

Sequences were aligned using the GeneBuilder Program and the aligned sequences were entered into the EBI website to carry out BLAST searches. The nucleotide sequence homology was the initial basis for identification of the genes.

6.2.4. Extension of DMC-1-M4 sequence

6.2.4.1. Isolation of fragments

The chromosomal DNA of DMC-1-M4 was restriction digested using the enzyme *EcoRI* as shown below:

DMC-1-M4 Chromosomal DNA preparation	30.0 μ l
10 X Buffer	4.0 μ l
dH ₂ O	5.2 μ l
<i>EcoRI</i> enzyme	0.8 μ l

Incubated at 37 °C for 30 min.

The restriction digestion fragments were extracted twice with phenol-chloroform followed by ethanol precipitation. DNA fragments were ligated in a dilute solution to favour self-ligation, with 100 μ l 5X T4 DNA ligase buffer and 1 μ l of T4 DNA ligase made up to 400 μ l with dH₂O. The solution was incubated at 16 °C overnight. The mixture was precipitated using 1 ml of 95 % ethanol and 16 μ l of 3 M sodium acetate at -70 °C for 15 min. DNA pellet was washed with 70 % ethanol, air-dried, resuspended in 20 μ l of dH₂O and used directly or stored at -20 °C.

6.2.4.2. Inverse PCR

Ligated DNA was used as the PCR template (Figure 6.1) with inverse primers to amplify the isolated fragment. The primers were Tn cat left and M4 Internal 4 forward (Appendix 2) the same as those previously employed in the sequencing of the DMC-1-M2 mutant. The PCR was carried out with the AccuPrime polymerase, which is a proof-reading polymerase efficient at extending long sequences. The reactions were set up in 0.5 ml microfuge tubes as follows:

2.5 μ l 10 X Buffer

0.2 μ l AccuPrime polymerase

19.55 μ l dH₂O

0.75 μ l Primer mix (diluted from stock solution to 20 μ M and mixed 1:1 to give final concentrations of 10 μ M).

The reactions were carried out on the DMC-1-M4 DNA ligations with positive and negative controls included. The samples were incubated at 95 °C for 2 min to activate the polymerase and separate DNA strands in the template. The samples were then subjected to 35 cycles of the following incubation sequence: denaturation at 95 °C for 30 sec, primer annealing at 50 °C for 30 sec, and product extension at 68 °C for 30 sec. Finally, the

product extension was maintained at 72 °C for 5 min. The thermocycling was carried out using a programmable dri-block (Hybaid OMN-E). The primers amplified the sequence to yield a PCR product that was cloned into a TOPO vector, which enabled the DMC-1-M4 sequence to be extended in one direction for further analysis.

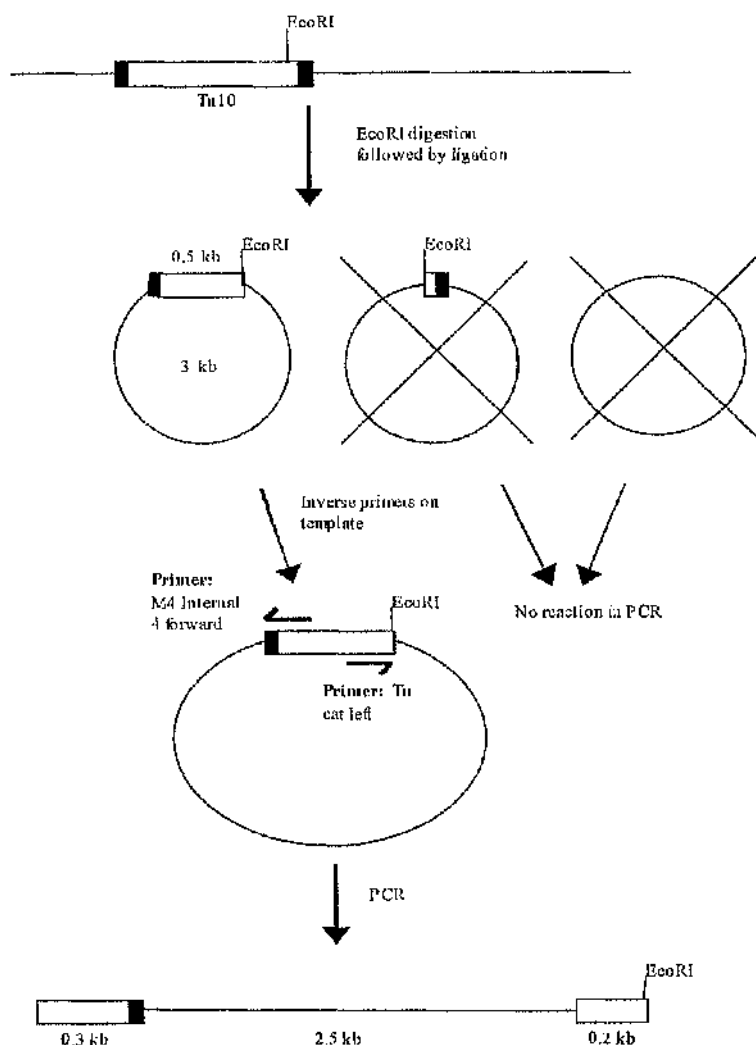


Figure 6.1. Template chromosomal DNA of DMC-1-M4 for inverse PCR. Chromosomal DNA was restriction digested with *EcoRI* enzyme to obtain fragments containing the partial transposon. These fragments were selected in PCR reactions using inverse PCR primers specific for the fragment of the transposon. PCR extended the sequence in one direction from the end of the transposon that was then aligned with previously obtained sequence.

6.2.4.3. TOPO cloning

All protocols were carried out in accordance with the Invitrogen 'TOPO TA Cloning Kit for Sequencing' manual (Appendix 8). AccuPrime polymerase is a proofreading polymerase which creates blunt-ended products and it was necessary to add 3' A-overhangs, again according to the Invitrogen manual (Appendix 8), to be able to clone the PCR fragments into the TOPO TA cloning vector. The resulting products were ligated into the TOPO TA vectors and heat-shock transformed into One Shot Chemically Competent *E. coli* TOP10 cells. The resultant *E. coli* clones were amplified through colony selection into 5 ml LB broth containing 50 µg ml⁻¹ ampicillin, and plasmids isolated for sequence determination as described previously.

6.2.5. Extension of *V. splendidus* DMC-1-M2 and M4 sequence.

From the extended sequence obtained for DMC-1-M4 it was identified that the transposon had inserted into a gene close to the insertion site of DMC-1-M2. Primers were designed to the end of both DMC-1-M2 and M4 that had matches with the same gene, to amplify the area of unidentified sequence between the two mutants.

6.2.5.1. PCR of unidentified sequence between DMC-1-M2 and M4.

Two sets of primer pairs were designed to amplify the unknown sequence between DMC-1-M2/DMC-1-M4 of >1 kb and >1.5 kb. These primers were designated DMC-1-M2/DMC-1-M4 forward and reverse 2 and DMC-1-M2/DMC-1-M4 forward and reverse 3 (Appendix 2). The PCR was set up as before using the AccuPrime polymerase system to amplify the unidentified sequence. The only change was the annealing temperature, which was increased from 50 °C to 52 °C to account for the different T_m of the primers.

6.2.6. Analysis of *V. splendidus* sequence containing the haemolysin gene

The sequences of DMC-1-M2 and DMC-1-M4 were aligned to cover an 8.7 kb section of the genome. The genes were identified and analysed using the EMBOSS program 'getorf', 'geecee' and 'palindrome' from the Bioinformatics service of the MRC RFCGR and programs from the EBI website. BioEdit was also employed to align sequences using ClustalW. The alignment of the haemolysin gene was carried out using the program DIALIGN. Phylogenetic trees were constructed using the PHYLIP package with the

maximum parsimony program PROTPARS. The data was bootstrapped with 100 replicates with a random input order to give the final consensus tree, which was visualised in the TreeView program.

6.2.7. PCR of the haemolysin gene

Primers located up and downstream of the start and stop codons were designed to amplify the complete haemolysin gene (Appendix 2), and PCR carried out as before with *Taq* polymerase. The amplified fragments were ligated into the TOPO TA cloning vector and transformed into *E. coli* TOP10 cells and the haemolytic activity of the clones was determined on blood plates containing 50 $\mu\text{g ml}^{-1}$ ampicillin. Clones were sequenced initially with M13 forward and reverse primers. Sequences were analysed as described previously.

6.2.8. Screening of *V. splendidus* strains for haemolysin gene

A selection of *V. splendidus* and *V. anguillarum* strains and a *Roseobacter* sp. were selected and their chromosomal DNA extracted using the BioRad kit, as before. The primers DMC-1 Hly 1 Reverse and DMC-1 Hly 1 Forward (Appendix 2) were employed to amplify an 800 bp fragment located within the haemolysin gene of *V. splendidus* DMC-1. The PCR was carried out on the chromosomal DNA preparations using the *Taq* polymerase protocol as before.

6.2.9. Comparison of the activity of *V. splendidus* haemolysin with that of aerolysin.

6.2.9.1. Haemolysin assay

A haemolysin assay was carried out to compare the activity of haemolysin with that of aerolysin. A freeze-dried sample of aerolysin was received as a gift from Prof. T. Buckley. Toxin was resuspended in 1 ml of dH_2O , divided into 50 μl amounts and stored at -20°C at a concentration of 100 $\mu\text{g ml}^{-1}$. The toxin was activated with trypsin prior to use, through cleavage of the carboxy-terminus according to Howard and Buckley (1985a). Toxin was diluted to 1.25 $\mu\text{g ml}^{-1}$ for comparison with *V. splendidus* haemolysin. The haemolysin assay conditions were described earlier (section 2.2.8 and 3.2.2).

6.2.9.2. Osmotic Protection Studies

Aerolysin was compared with *V. splendidus* haemolysin (bacterial supernatant) in a haemolysis osmotic protection study using assay conditions described previously (Chapter 5, section 5.24).

6.3. Results

6.3.1. DNA sequence surrounding the transposon insertion site in DMC-1 mutant DMC-1-M2.

As described in chapter 5 the DNA sequence surrounding the transposon insertion site was determined for a region covering 3.7 kb of *V. splendidus* DNA sequence. Complete sequence data is shown in Appendix 3 and 4, and sequences were aligned using GeneBuilder. From this, the consensus sequence was subjected to a BLAST search (EBI website), which indicated nucleotide sequence homology with genes for the sodium/alanine symporter and hypothetical proteins of *V. cholerae*, *V. vulnificus* and *V. parahaemolyticus*. No haemolysin genes were identified in this DNA sequence.

6.3.2. DNA sequence surrounding the transposon insertion site in DMC-1 mutant DMC-1-M4.

Over 10,000 transposon mutants were screened to obtain an additional haemolysin negative mutant that was designated DMC-1-M4. The nucleotide sequence of the region containing the transposon insertion was determined in both directions to cover an approximate 2 kb fragment of *V. splendidus* genomic DNA (excluding the transposon). An initial BLAST search showed homology with the gene for aerolysin from several *Aeromonas* spp. but other parts of the sequence showed no identity to sequences in the EMBL database.

The nucleotide sequence closest to the DMC-1-M4 transposon insertion site was extended to identify more of the genes located both up and downstream of the haemolysin gene. This was done by inverse PCR on *Eco*RI restriction digestion fragments containing the partial transposon of the mutant DMC-1-M4. The amplified fragments were ligated into TOPO Cloning vectors and the full insert was sequenced to cover the full 3.0 kb fragment (Figure 6.1 and Appendix 4) using primers subsequently designed against the sequence obtained. These sequences extended the DMC-1-M4 insert by an additional 2 kb, as shown in Figure 6.2. The sequences were aligned accordingly using the GeneBuilder program alongside the previously obtained sequence. The nucleotide sequence had homology with

hypothetical proteins and a carboxypeptidase from *Vibrio* spp. located upstream from the identified genes with homology of the DMC-1-M2 sequence.

6.3.3. Extension of *V. splendidus* DMC-1-M2 and M4 sequence.

The sequences from the two mutants were successfully overlapped using primers designed against the appropriate end of each sequence (Figure 6.2), with an additional 1 kb of sequence being obtained that fully aligned the DMC-1-M2 and DMC-1-M4 sequences (Appendix 4).

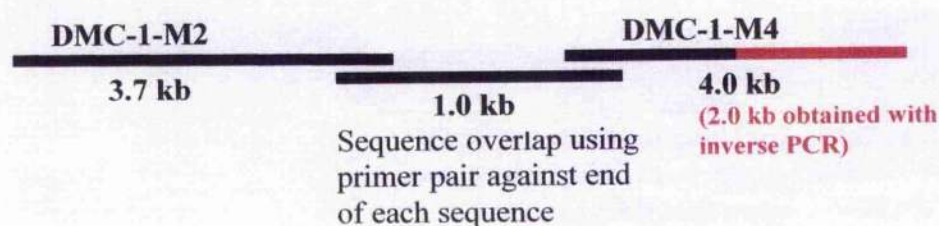


Figure 6.2. Schematic diagram of the overlap of DMC-1-M2 and M4. Primers were designed against the end of each sequence to extend the sequence over the unidentified region to enable both DMC-1-M2 and M4 to be aligned to yield an 8.7 kb fragment of the *V. splendidus* genome. The section on DMC-1-M4 highlighted in red indicates the area of sequence obtained using inverse PCR.

6.3.4. Sequence analysis of the full 8.7 kb fragment of *V. splendidus* genome.

The full 8.7 kb DNA sequence (Appendix 6) covering the region of transposon insertion in the DMC-1-M2/DMC-1-M4 mutants was analysed using the Bioinformatics software EMBOSS on the MRC RFCGR website. The sequence was analysed for open reading frames (orfs) using the program 'getorf' and from the whole sequence only 12 orfs were identified. From BLAST searches significant matches with known genes were identified for only 5 orfs (Table 6.1; only the top three hits are shown). The sequence of interest was annotated using the Artemis program from the Sanger Centre website (Figure 6.3).

The annotation in Figure 6.3 shows the orfs that were identified, with the vertical bars representing stop codons. Significant genes are coloured in red, and also annotated is the promoter region of the haemolysin (hereafter named splendilysin) containing the Shine

Dalgarno sequence, -10 site (TATA box) and the -35 site. These are shown in more detail in Figure 6.4.

Figure 6.5 provides an overview of the gene arrangement in *V. splendidus* in comparison to the genes of other *Vibrio* species. It is clear from Figure 6.5 that a possible DNA insertion has occurred involving the haemolysin gene. Further evidence for this is that the *Vibrio* genes are homologous for all four species and there is an almost identical alignment at either side of this proposed insertion. The insertion seems to have occurred within or after the sodium/alanine symporter gene and after the gene for the hypothetical/putative threonine efflux protein. The insertion covers orfs 3 and 7 that have homology with transcriptional activator, ToxR genes (Figure 6.6) and aerolysin genes (Figure 6.8).

Also shown in Figure 6.5 are the transposon insertion sites identified in the DMC-1-M2 and DMC-1-M4 mutants, both of which have occurred within the proposed inserted DNA element, resulting in haemolysin-negative phenotypes. The DMC-1-M2 mutant contained the transposon inserted within the ToxR element, which is possible evidence that this is a transcriptional factor involved in the expression of the haemolysin. The DMC-1-M4 transposon insertion has occurred directly within the haemolysin gene.

The region of the sequence with high homology to ToxR includes residues 1-170 of the *V. cholerae* ToxR gene (Figure 6.6) which has been identified as the cytoplasmic domain with a helix-turn-helix (HTH) structure. This HTH motif is followed by a wing composed of two β -strands that are separated by a loop (Crawford *et al.*, 2003). This region is a DNA binding/transcription activation domain that is common in a variety of prokaryotic and eukaryotic regulators (Crawford *et al.*, 2003). The *V. splendidus* gene sequence was analysed using ProteinPrediction program and was found to have regions that contain both beta sheets and helices (Figure 6.7). The same analysis was carried out on the *V. cholerae* ToxR sequence as a comparison (Figure 6.7).

6.3.4.1. Identification of the inserted element containing the haemolysin gene.

Figure 6.5 clearly identifies that an insertion has occurred in the *V. splendidus* genome with the ToxR and aerolysin homologous genes. An insertion has also occurred in the *V.*

cholerae genome. To identify the possible sites at which DNA insertion has occurred in the *V. splendidus* genome, the sequences prior to and after the ToxR and the haemolysin genes were analysed using the EMBOSS program 'palindrome' to identify inverted repeats in the nucleotide sequence. Inverted repeats were found between the haemolysin orf and the subsequent gene (orf 10), and either before the ToxR gene or directly before the start of the haemolysin (Figure 6.3). The longest sequence inverted repeat found was 12 bp, whereas such sequences are usually around 18 bp (Kleckner, 1981).

The presence of inverted repeats suggests that this sequence of DNA may have become inserted as a result of genetic mobilisation by a transposon element or by phage integration. Another characteristic of inserted DNA in a sequence is manifested by a change in GC content compared to the genes in the rest of the sequence. The GC content of each orf was measured using the 'geecee' program in EMBOSS (Table 6.1). There were no significant differences between orfs 3 and 7 compared with the rest of the orfs present in this section of DNA sequence. The lowest GC content was 0.37 and was for orf 8, which is located between orf 9 and 10 and has no homology with any genes in the BLAST database. Another way to identify DNA of different origin is to look at the codon usage in the genes of interest. However, the proposed inserted DNA probably originates from either *Vibrio* or *Aeromonas*, both of which belong to the Vibrionaceae family. Therefore, codon usage would be similar and this analysis was not considered worthwhile.

Open Reading Frame (orf)	Nucleotides (nt)	Length (nt)	GC content	Gene matches (Top hits)	E-value	Identity	Length of gene
1	2690-2929	239	0.48	No hits	-	-	-
2	1812-3017	1205	0.48	Sodium/alanine symporter (<i>Vibrio parahaemolyticus</i>)	0.0	81 % (331/401)	458 nt
				Sodium/alanine symporter (<i>Vibrio vulnificus</i> CMCP6)	0.0	81 % (331/404)	458 nt
				Sodium/alanine symporter (<i>Vibrio cholerae</i> O1 biovar)	0.0	79 % (322/404)	458 nt
3	3574-4083	509	0.42	Putative transcriptional activator ToxR (<i>Vibrio parahaemolyticus</i>)	6e-10	30 % (41/135)	183 nt
				ToxR (<i>Serratia marcescens</i>)	2e-7	29 % (49/164)	279 nt
				Cholera toxin transcriptional activator (<i>Vibrio cholerae</i> O1 biovar)	2e-7	29 % (49/164)	294 nt
4	4380-4607	227	0.44	Putative cytochrome P450 (<i>Oryza sativa</i> (japonica cultivar-group))	2.7	35 % (14/39)	520 nt
5	4576-4836	260	0.41	E3A like HECT domain containing ubiquitin protein ligase (<i>Cryptosporidium parvum</i>)	6.0	32 % (15/46)	1561 nt
				Unknown (environmental sequence)	7.9	37 % (20/53)	273 nt
6	6397-6819	422	0.48	Unknown (environmental sequence)	4.6	40 % (14/35)	195 nt
7	5376-6869	1493	0.46	Haemolysin (<i>Aeromonas hydrophila</i>)	e-150	54 % (259/471)	488 nt
				Haemolysin (<i>A. sobria</i>)	e-150	55 % (260/471)	488 nt
				Proaerolysin	e-148	53 % (253/470)	470 nt
8	7602-7841	239	0.37	No hits	-	-	-
9	7935-8684	749	0.48	Thermotable carboxypeptidase 1 (<i>Vibrio cholerae</i>)	e-110	76 % (190/250)	524 nt
				Zinc-dependent carboxypeptidase (<i>Vibrio vulnificus</i> CMCP6)	e-108	75 % (188/249)	490 nt
				Zinc-dependent carboxypeptidase (<i>Vibrio vulnificus</i> YJ016)	e-107	75 % (187/249)	502 nt

10	7505-6993 (reverse sense)	512	0.46	Conserved hypothetical protein (<i>Vibrio parahaemolyticus</i>)	9e-68 Significant	85 % (126/148)	211 nt
				Putative threonine efflux protein (<i>Vibrio vulnificus</i> YJ016)	6e-66 Significant	81 % (122/149)	211 nt
				Putative threonine efflux protein (<i>Vibrio vulnificus</i> CMCP6)	8e-66 Significant	81 % (122/149)	211 nt
11	2755-2417 (reverse sense)	338	0.47	Hypothetical protein (<i>Agrobacterium tumefaciens</i> strain C58)	7.8 Not significant	23 % (16/69)	177 nt
12	504-217 (reverse sense)	287	0.41	No hits	-	-	-

Table 6.1. Analysis of the 8.7 kb fragment from the *Vibrio splendidus* genome. Details of open reading frames located within the 8.7 kb fragment of the *V. splendidus* genome

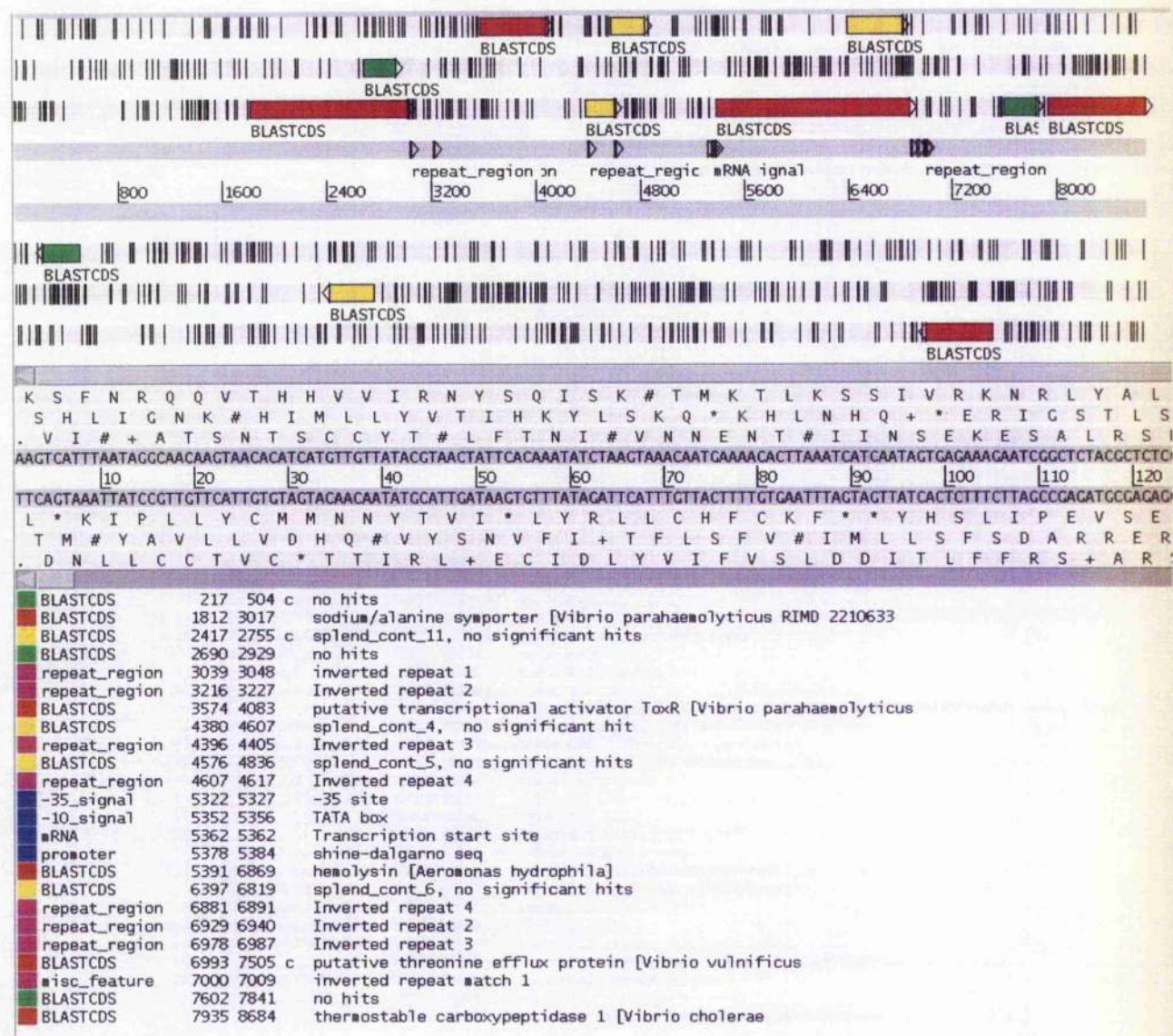


Figure 6.3. Annotation of *V. splendidus* DNA sequence using the Artemis program from the Sanger Centre website to annotate the sequence. Open reading frames (orfs) in the three forward and three reverse reading frames are shown, the legend below reads on diagram from left to right with nucleotide numbers and gene identification indicated. Orfs with significant matches are highlighted in red, all orfs are described in Table 6.1.

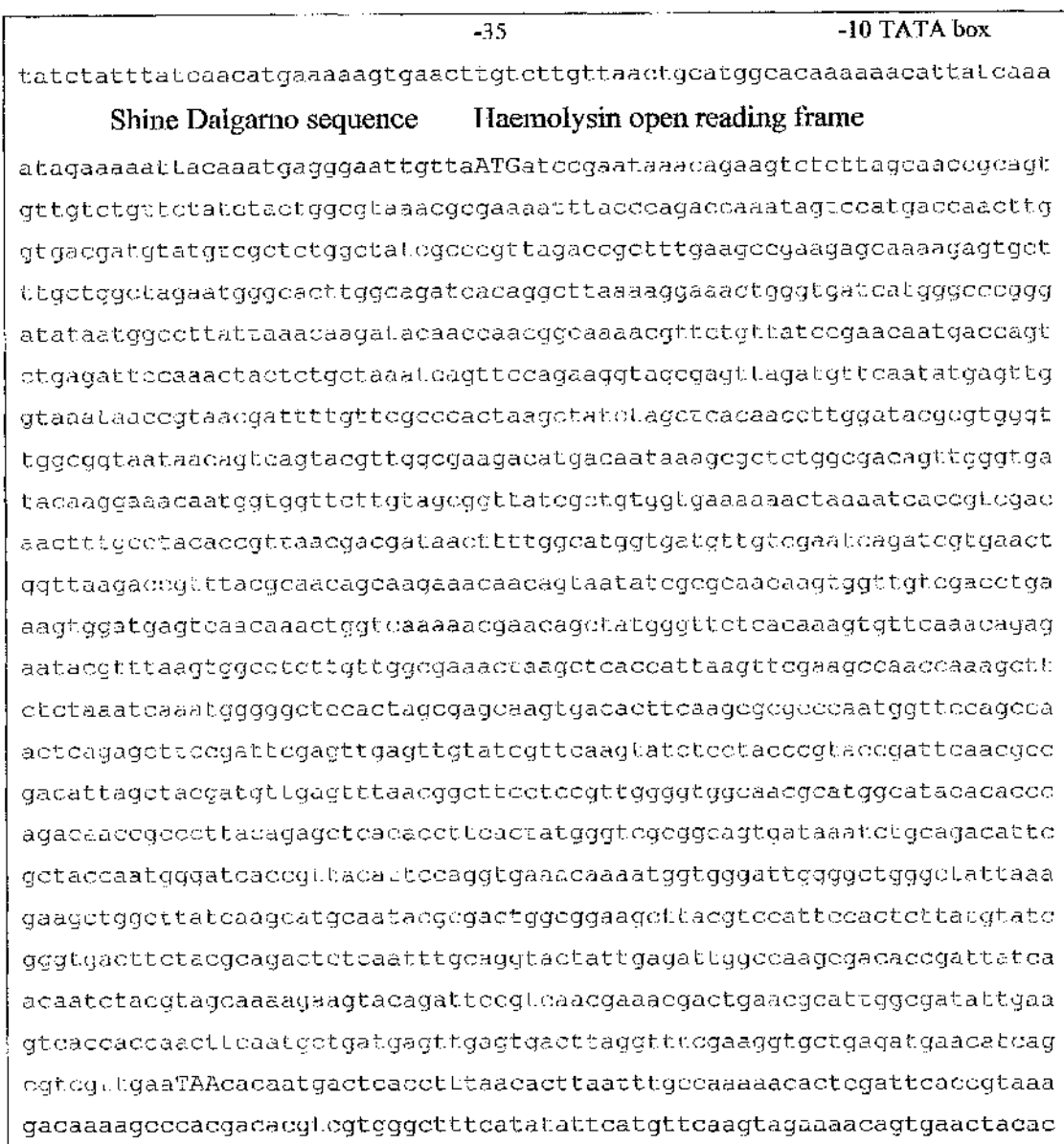


Figure 6.4. Promoter sequence of the haemolysin orf 7. The putative Shine Dalgarno sequence, -35 and -10 sequences are shown in blue and the putative haemolysin gene is shown in red.

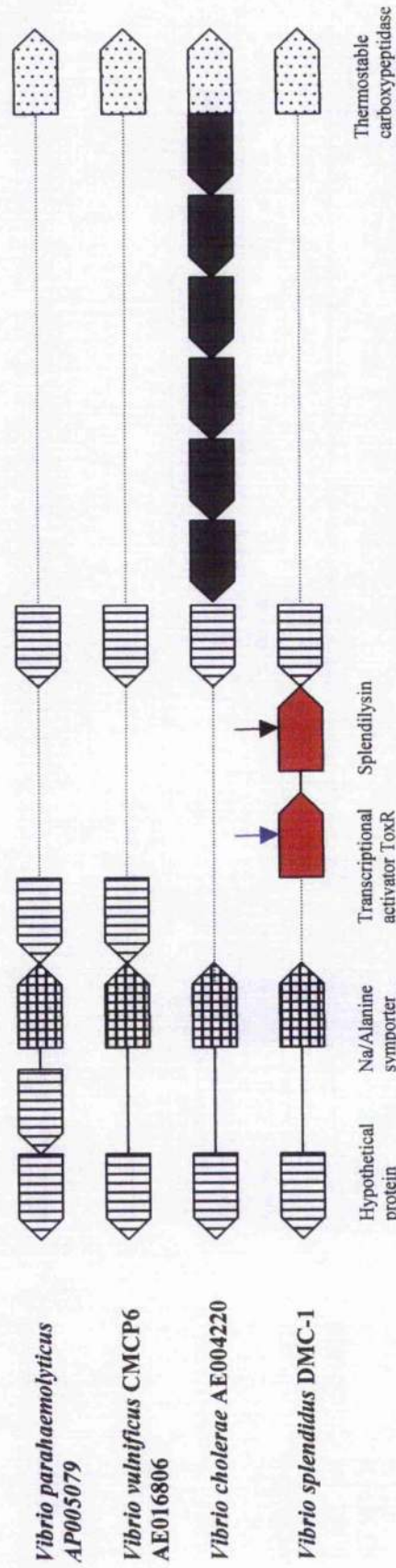


Figure 6.5. Comparison of homologous genes in the genomes of *Vibrio* spp. The genes covered are: *V. parahaemolyticus* VP1739, Hypothetical protein; VP1740, Hypothetical protein; VP1741, Sodium/alanine symporter; VP1742, Hypothetical protein; VP1743, Conserved Hypothetical protein; VP1744, Thermostable carboxypeptidase. *V. vulnificus*: VV12693, Chromosome segregation ATPase; VV12695, Sodium/alanine symporter; VV12696, Unknown; VV12697, Putative threonine efflux protein; VV12698, Thermostable carboxypeptidase. *Vibrio cholerae*: VC1423, Hypothetical protein; VC1422, Sodium/alanine symporter; VC1421, Conserved hypothetical protein; VC1420, Hypothetical protein; VC1419, Hypothetical protein; VC1418, Hypothetical protein; VC1417, Hypothetical protein; VC1416 vgrG protein; VC1415, hcp protein; VC1414, Thermostable carboxypeptidase 1. *Vibrio splendidus* DMC-1: VS 1, Hypothetical protein; VS2, Sodium/alanine symporter; VS 3, transcription activator ToxR; VS 4, Splendilysin; VS 5, hypothetical protein; VS 6, thermostable carboxypeptidase. The dashed lines indicate the areas where insertions have occurred in either *V. cholerae* or *V. splendidus*, indicated with the black and red genes.

↓ DMC-1-M2 transposon insertion ↓ DMC-1-M4 transposon insertion

6.3.5. Cloning of the splendilysin gene

Four clones were selected for sequence analysis, two of which had haemolytic phenotypes, the other two being haemolysin negative. Partial sequences were obtained (Appendix 5) and overall there was alignment with the haemolysin consensus. However, some nucleotide differences occurred compared to the consensus and these are highlighted in red (Appendix 5). There were no obvious differences between the sequences of the haemolysin-positive and -negative clones.

6.3.6. *Vibrio splendidus* strains

A range of *V. splendidus* strains was screened using PCR to assess the occurrence of the haemolysin gene in this species. The primers employed amplified an 800 bp region of the haemolysin gene and for the PCR it was necessary to use chromosomal DNA extracted using a commercial kit, as templates because heat-treated cells failed to yield products with this primer pair.

Table 6.2 gives an overview of the strains tested and their characteristics. The haemolytic *V. splendidus* biovar 1 strains gave positive results (Figure 6.9) but LMS strains, with a haemolysin-negative phenotype, and *V. splendidus* biovar 2 were negative for the gene, as were the negative control strains of *V. anguillarum* and *Roseobacter* spp. There was no difference in detection of the haemolysin gene between the strains previously found to be virulent and avirulent against turbot larvae (Thomson, 2001) as both types contained the gene.

Type	Strain	Virulence towards turbot larvae ¹	Haemolytic titre ²	PCR 800bp product ³	Sucrose utilisation ⁴
91079	<i>V. anguillarum</i>	Virulent ⁵	N.D.	-	+
DMC-1	<i>V. splendidus</i> bv 1.	Virulent	Yes (1/256)	+	-
LTS-3	<i>V. splendidus</i> bv 1.	Virulent	Yes (1/256)	+	-
LTS-4	<i>V. splendidus</i> bv 1.	Avirulent	Yes (1/128)	+	-
HNF-8	<i>V. splendidus</i> bv 1.	Virulent	Yes (1/256)	+	-
LTH-4	<i>V. splendidus</i> bv 1.	Avirulent	Yes	+	+
DMC-2	<i>V. splendidus</i> bv 1.	N.D.	Yes	+	-
DTC-5	<i>V. splendidus</i> bv 1.	Virulent/Avirulent ⁶	Yes (1/256)	+	-
DTR-2	<i>V. splendidus</i> bv 1.	Avirulent	Yes	+	-
DTY-1	<i>V. splendidus</i> bv 1.	Avirulent	Yes (1/32)	+	+
LMS-1	<i>V. splendidus</i> bv 1.	Avirulent	No (-)	-	+
LMS-2	<i>V. splendidus</i> bv 1.	N.D.	No (-)	-	+
LMS-3	<i>V. splendidus</i> bv 1.	N.D.	No (-)	-	+
LMS-4	<i>V. splendidus</i> bv 1.	N.D.	No (-)	-	+
LTH-1	<i>V. splendidus</i> bv 2.	Avirulent	Yes	-	+
LTH-3	<i>V. splendidus</i> bv 2.	Avirulent	Yes (1/2)	-	+
HNF-1	<i>Roseobacter</i> spp.	Avirulent	N.D.	-	N.D.

Table 6.2. Screening of strains for the presence of haemolysin gene. A range of *V. splendidus* strains and control strains *V. anguillarum* and *Roseobacter* spp. were screened for the haemolysin gene using PCR, other characteristics of these strains are listed.

¹ Larval trials carried out by Thomson (2001)

² Haemolysin measured using blood agar plates and the titre measured in the haemolysin assay

³ Haemolysin gene partially amplified using PCR primers designed against an 800 bp sequence from the identified splendilysin sequence

⁴ Sucrose utilisation measured on TCBS plates

⁵ *Vibrio anguillarum* 91079 was shown to be virulent for turbot larvae by Munro *et al.* (1995)

⁶ *Vibrio splendidus* DTC-5 was shown to be virulent in one turbot larval trial and avirulent in two subsequent trials (Thomson, 2001).

<i>Vibrio anguillarum</i>	1	-----MSNIGTKFILANRFTFFNSRSLLEQENEQEAUPLGNSNESHYLLQLCE	48
<i>Vibrio parahaemolyti</i>	1	-----MTNIGTKFLLAQRFTFFPNSNSIADQQSGNEVRLGNSNESHYLLQLCE	48
<i>Vibrio cholerae ToxR</i>	1	MFGLGHNSKEISMSHIGTKFILAEKFFFDPLSNLIDKEDSEETPLGNSNESHYLLQLCE	60
<i>Serratia marcescens</i>	1	-----KEISMSHIGTKFILAEKFFFDPLSNLIDKEDSEETPLGNSNESHYLLQLCE	52
<i>Vibrio fischeri ToxR</i>	1	-----MNTENNKKIIIGDRFLFDPLNPSLIDTLNDEETPLGNSNESHYLLQLCE	48
<i>ToxR Vibrio splendid</i>	1	-----MIRFDEIKNSFYNDERS---IKIGYRETFVLLQLL	33
<i>Vibrio anguillarum</i>	49	RPNEVLPFNELEHEFVREQGFVETISSEITCATSTLFFMLKDQTKSPEFRTVPERGYCMI	108
<i>Vibrio parahaemolyti</i>	49	RPNEVLPFNELEHEFVREQGFVETISSEITCATSTLFFMLKDQTKSPEFRTVPERGYCMI	108
<i>Vibrio cholerae ToxR</i>	61	RPNEVLPFNDLEHDFVREQGFVETISSEITCATSTLFFMLKDQTKSPQYRTVPERGYCMI	120
<i>Serratia marcescens</i>	53	RPNEVLPFNDLEHDFVREQGFVETISSEITCATSTLFFMLKDQTKSPQYRTVPERGYCMI	112
<i>Vibrio fischeri ToxR</i>	49	EPKVVVEREEHEFVARDQGFVETISSEITCATSTLFFMLQDPTKLPVYRTVPERGYCMI	108
<i>ToxR Vibrio splendid</i>	34	NSPEINVECDISEFAG--SEFISITSLACISLLRQGFVKLGIRETTIVTVEKVGRTI	91
<i>Vibrio anguillarum</i>	109	AS-VTRATFYSQK--NTTNEPEEEPELLASESIQDIDADTTTSQPEETTSQTVTITDEPLAKVK	165
<i>Vibrio parahaemolyti</i>	109	CT-VERLSPLSS---DSSSIEVEEPASDNDASANEVETIVEFSLATTSDAIVEPEAFVV	164
<i>Vibrio cholerae ToxR</i>	121	AR-VETVEEEMAR--ES-----EAAHDISQESVNEYAESSSVSSATVVNTFQPANVV	171
<i>Serratia marcescens</i>	113	AR-VETVEEEMAR--ES-----EAAHDISQESVNEYAESSSVSSATVVNTFQPANVV	163
<i>Vibrio fischeri ToxR</i>	109	STAVAQVDQDQTEAETENHLENTFSIDSLNTASVHODDAQEAQAVAFQCNATINQPSSTT	168
<i>ToxR Vibrio splendid</i>	92	DE-CVFIDSQPC-----SEVTIPLTFVS-----	113
<i>Vibrio anguillarum</i>	166	IGKEKLTQS-----KLHLVVALFLPILALLLTSPAQSKFKQIAVYNNIVNTPRHP	218
<i>Vibrio parahaemolyti</i>	165	PEKAPVASAVNFWIP--RVILFLALLLPICVLLFTNPAESQFRQIGEQYCNVVTTPVNH	222
<i>Vibrio cholerae ToxR</i>	172	TNKSAPNLGN-----RLILIAVLLPLAVLLLTNFSQTSFKPLTVVDGVAVNMPNHP	224
<i>Serratia marcescens</i>	164	ANKSAPNLGN-----RLILIAVLLPLAVLLLTNFSQTSFKPLTVVDGVAVNMPNHP	216
<i>Vibrio fischeri ToxR</i>	169	NSTPQAHLSLLG-----TIMLIAPVLPPIAYVNHTFKSDAFVEVANYDNTPVFVPH	223
<i>ToxR Vibrio splendid</i>	113	---EQVELGQ-----S---NIAASIASNDT	132
<i>Vibrio anguillarum</i>	219	DLNKLWPSIEKCVKKNESHTGELAPKQVIATGGQNNQLVNVIHSSEFSGENVTLRILA	278
<i>Vibrio parahaemolyti</i>	223	QINNWLPSIEQCIERVKKHHAEDSLPVEVIATGGQNNQLINVIHDSNHSYENVTLRIFA	282
<i>Vibrio cholerae ToxR</i>	225	DLNKLWPSIELCVKKNKHKHTGGLKPIEVIATGGQNNQLINVIHSPFVSGENITLRIVA	284
<i>Serratia marcescens</i>	217	DLNKLWPSIELCVKKNKHKHTGGLKPIEVIATGGQNNQLINVIHSPFVSGETIPSRNCG	276
<i>Vibrio fischeri ToxR</i>	224	SIERWKFLEIQCTNPNNSKHDTSLKPIEVIATSGQPNQITINVIHSEEHSDQISVRLLI	283
<i>ToxR Vibrio splendid</i>	133	PESKSTSLTYSQVSLKRNRLCYFITGLFVVLG-----LSEFC-----	170
<i>Vibrio anguillarum</i>	279	NQD-----DLANVCK-----	288
<i>Vibrio parahaemolyti</i>	283	GQN-----DPTDICK-----	292
<i>Vibrio cholerae ToxR</i>	285	NFN-----DAIKVCE-----	294
<i>Serratia marcescens</i>	276	-----VLK-----	279
<i>Vibrio fischeri ToxR</i>	284	DQKGWIRYVDKORAVTSIQTFITLNPANYLSNR	316
<i>ToxR Vibrio splendid</i>	170	-----	170

Figure 6.6. Alignment of ToxR amino acid sequences. The *V. splendid* DMC-1 ToxR homologue amino acid sequence was aligned with the known ToxR sequences from the EMBL database, using ClustalW package in the BioEdit program. Blocks of colour identify functional homology of sequences.


```

VS ToxR      1  MIRFDPIKNRIYNDERSIKIGYRETRVLELLLNKSPEIVNKQDIISFAWGSEFIGDTSLAKCISLLRQGFVKLGIRETPIVTPKVGYRLIDECV 95
VC ToxR      1  MFGLGHNSKEISMESHIGTKFILAEEKFTFDPLSNTLIDKEDSEEIIRLGSNESRIILWLLAQRPNEVISRNDLHDFVWREQGFEVDDSLTQAISTLRKMLKDSTKSPQYVKTVPKR 115

VS ToxR     96  FDSQPQSEVTIPLTPVSEQVPLGOSNIA ASIANDTPESKSTSLTYSQVSLYKNRLCYFITGLFIWLGIGFC 170
VC ToxR    116  GYQLIARVETVEEEMARESEAAHDISQPEVNEYAESSVPSSATVNTPQANVVTNKSAPNLGNRLLULLIAVLPLAVILLTNPSQTSFKPLTWVDGVAVNMPNHPDLSNW 229
               .....
VC ToxR    230  LPSIELCVKKYNEKHTGGLKPIEVATGGQNNQLTLNYIHSPEVSGENITLRIVANPNDAIKVCE 294

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Figure 6.7. Structural analysis of the ToxR amino acid sequence from *V. splendidus* and *V. cholerae*. **VS ToxR**, *V. splendidus*; **VC ToxR**, *V. cholerae*.

The structural predictions were carried out using the profile network prediction HeiDelberg, The PredictProtein server. The amino acids highlighted in blue represent the beta sheets and the red highlight the helical regions. The amino acids marked with * are predicted to represent a transmembrane domain.

Amino terminus cleavage site
↓

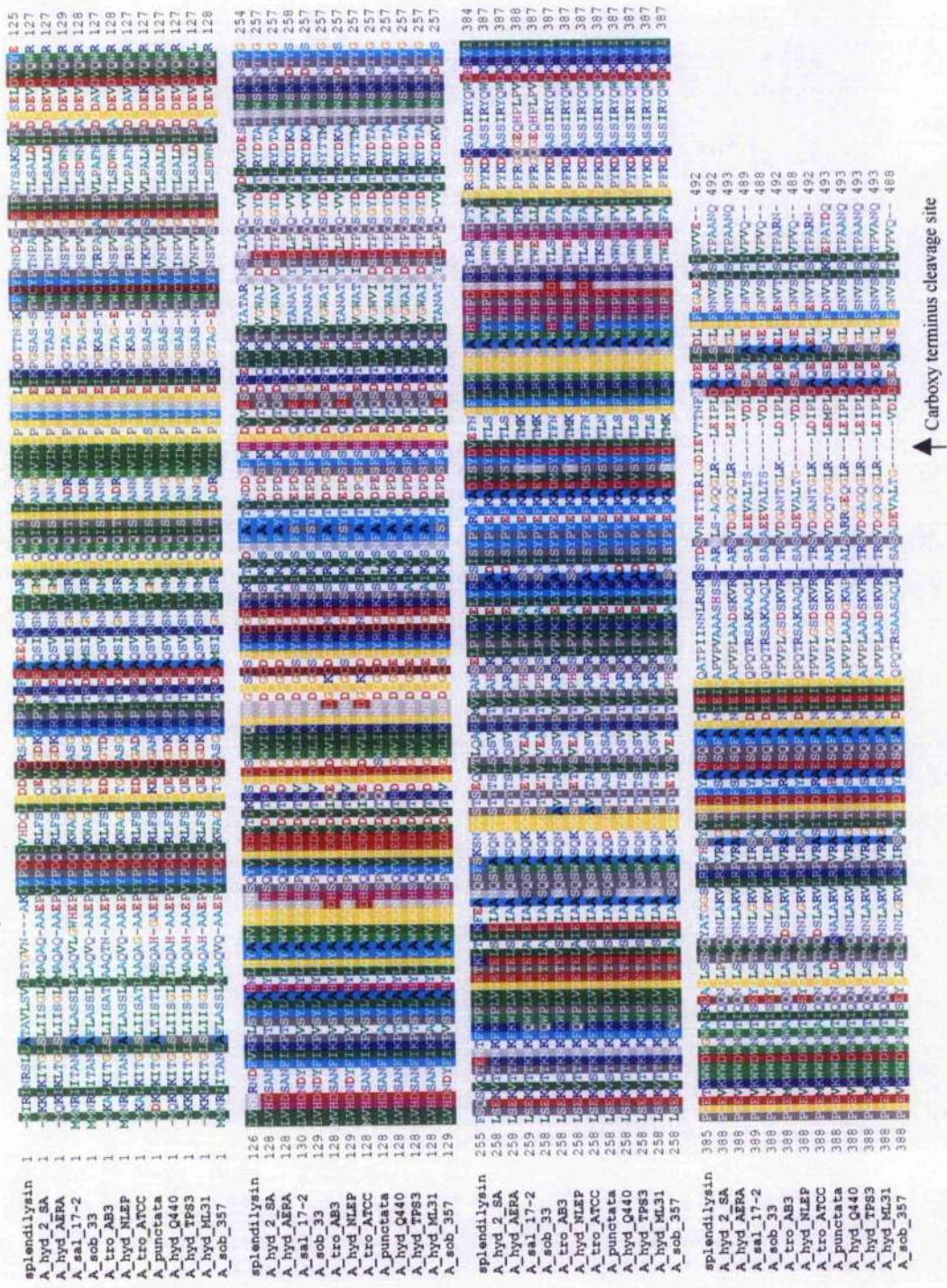


Figure 6.8. Alignment of aerolysin and splendilysin using BioEdit program to view. Amino acid sequences of aerolysins from EMBL were aligned with splendilysin using DIALIGN program and were annotated in BioEdit. Arrows indicate the regions of aerolysin that undergo cleavage during processing of toxin. Blocks of colour identify regions with functional homology.

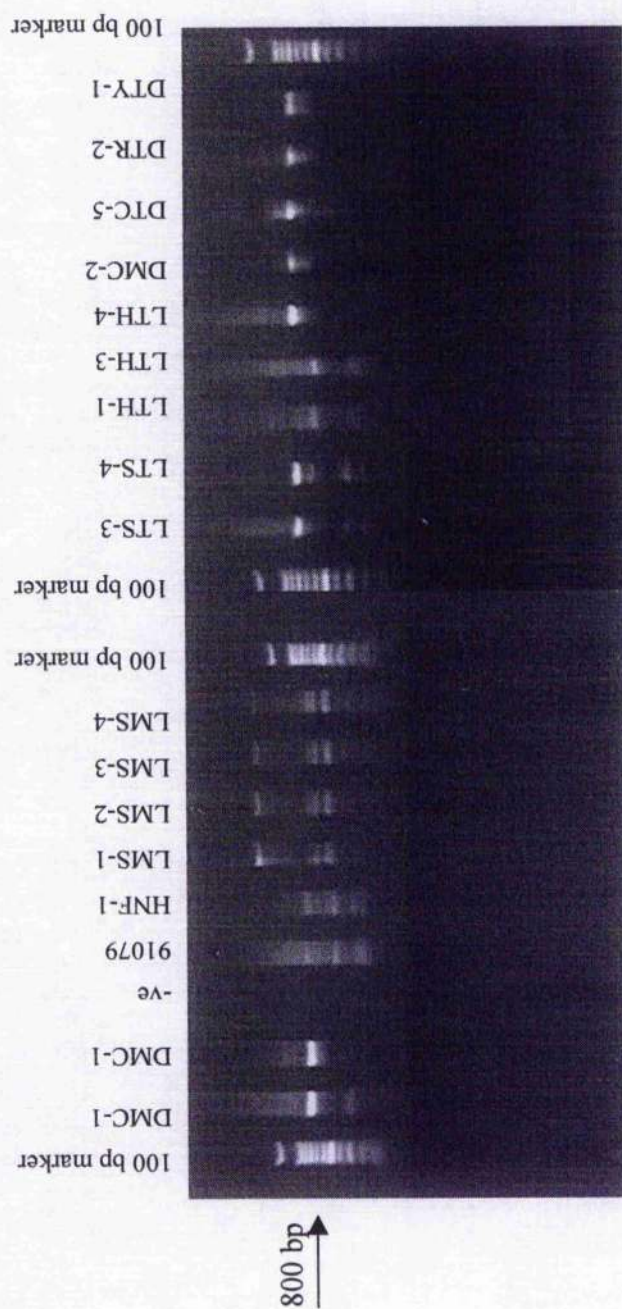


Figure 6.9. PCR products of haemolysin formed using primers to an 800 bp section of the splendilysin gene using DNA from *V. splendidus* strains DMC-1-2; LMS-1-4; LTS-3-4; LTH-1, 3, 4; DTC-5, DTR-2 and DTY-1, *V. anguillarum* 91079 and *Roseobacter* HNF-1 (Arrow indicates the PCR product at 800 bp). Gel electrophoresis of the PCR products for the strains listed in Table 6.2 in a 0.7 % agarose gel.

6.3.7. Comparison of the splendilysin and aerolysin amino acid sequences

The *V. splendidus* splendilysin gene (Appendix 7) was identified as having homology with aerolysin genes (Table 6.1 and Figure 6.3) and the splendilysin amino acid sequence was aligned with 12 aerolysin amino acid sequences using the DIALIGN program from the MRC RFCGR Bioinformatics Applications (Figure 6.8). The amino acids were aligned and homologous regions were colour coded with areas of 100 % functional homology highlighted in blocks of colour. For the aerolysin group of sequences the cleavage site of the signal peptide at the amino terminus is indicated in Figure 6.8 and occurs between the two alanine residues. At the carboxy terminus there is a cleavage site between arginine and leucine, that is known to activate proaerolysin into the active aerolysin toxin. However, as shown in Figure 6.8 the sequence for the *V. splendidus* splendilysin at these cleavage sites varies from that of the aerolysin group. These differences may result in splendilysin being processed in a different manner to that of aerolysin.

The amino acid alignments for the toxin gave the final result shown in the consensus tree in Figure 6.10. These results show that the aerolysins of the *Aeromonas* spp. form two clusters with the *V. splendidus* splendilysin being quite different from each cluster, although still having homology with both.

6.3.7.1. Osmotic protection studies with aerolysin.

After identification of splendilysin as a homologue of aerolysin a sample of *A. hydrophila* aerolysin was obtained from Prof. Thomas Buckley for comparison with splendilysin. The two haemolysins were compared in the osmotic protection assay using toxin preparations of similar haemolytic activity (aerolysin titre 1/256 at $1\mu\text{g ml}^{-1}$). The two preparations yielded similar dose response curves in haemolytic assays using sheep erythrocytes (Figure 6.11).

The osmotic protection study was carried out with three different concentrations of aerolysin (Figure 6.12) enabling the diameter of the membrane pore to be estimated (Figure 6.13). The estimated radius of the pore was 0.9-1.0 nm, compared with 0.8-0.9 nm for splendilysin (Chapter 5).

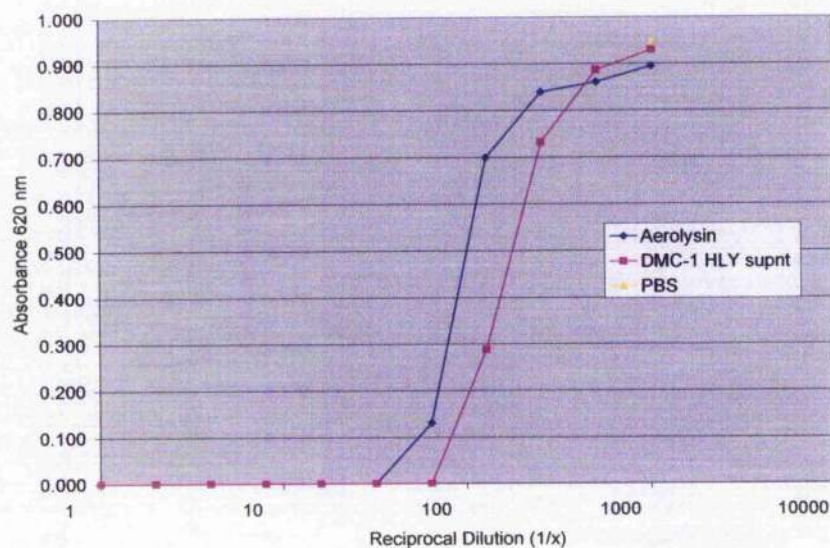


Figure 6.11. Comparison of splendilysin and aerolysin using the haemolysin assay. Doubling dilutions of both toxin preparations (neat-1/1026) were titrated in the haemolysin assay with 1 % washed sheep blood. Haemolytic activity was measured using cell scattering data at an absorbance of 620 nm. PBS was used as a negative control.

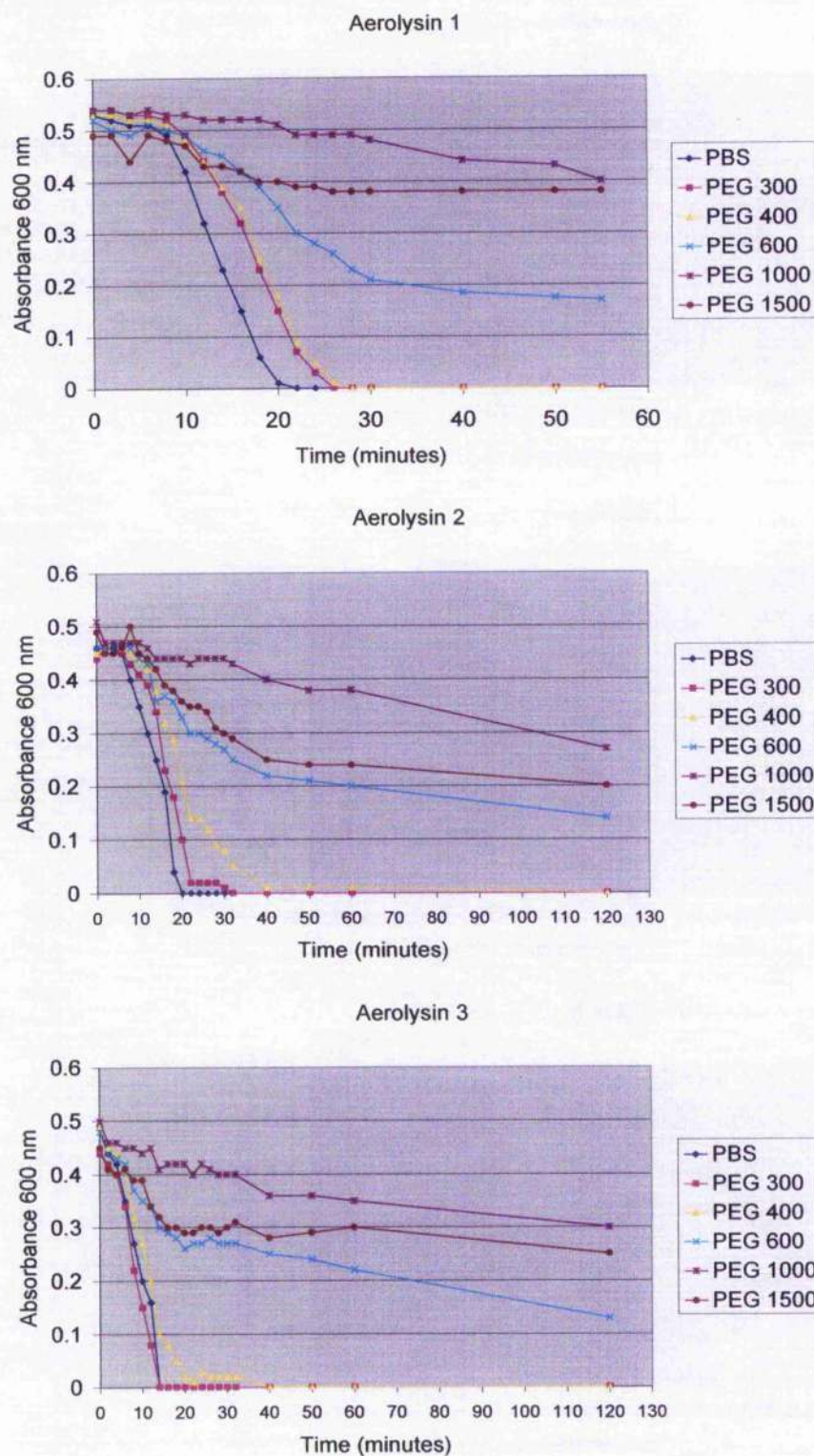


Figure 6.12. Osmotic protection studies with aerolysin toxin. Toxin treated erythrocytes were suspended in a series of PEG solutions in three separate experiments to measure osmotic protection effects of these solutions. PBS was added to toxin-treated erythrocytes to act as a positive haemolytic control. Haemolytic activity was measured using cell scattering data at an absorbance of 620 nm.

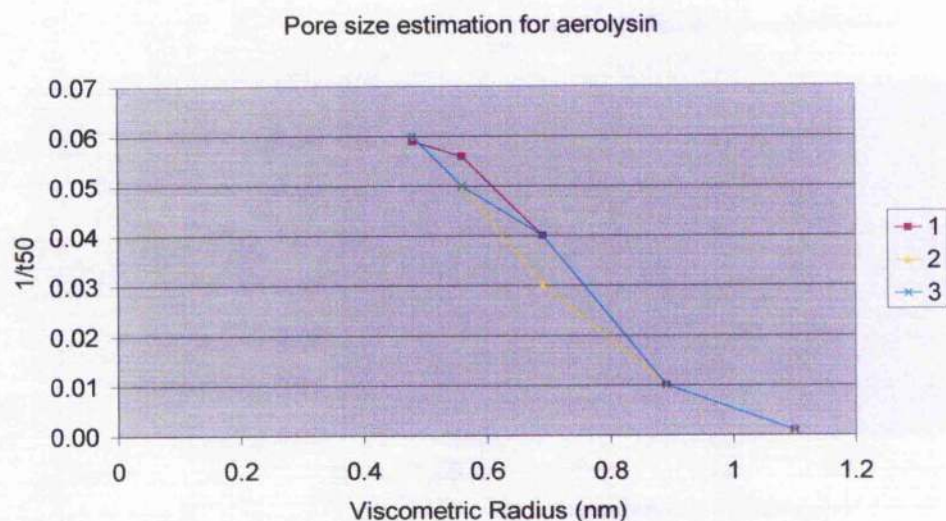


Figure 6.13. Estimation of aerolysin toxin pore-size. The average $1/t_{50}$ data of the PEG solutions from Figure 6.12 was plotted against their known viscometric radii to estimate the membrane channel radius formed by aerolysin.

6.4. Discussion

The inactivation of splendilysin was important to assess its importance as a virulence factor for *V. splendidus* DMC-1. As discussed in chapter 5 the virulence of *V. splendidus* was attenuated with the transposon mutants, DMC-1-M2 and DMC-1-M4, suggesting that the haemolysin does play an important role in the virulence of this organism. For *V. vulnificus* the opposite was found in a study by Massad *et al.* (1988) where non-haemolytic mutants were as virulent as their parent strain. Another example is the mutants of *A. salmonicida* that were deficient in production of the lethal toxins glycerophospholipid cholesteryl acyl transferase and serine protease, but were still lethal to fish (Vipond *et al.*, 1998). Therefore, cytotoxins are not always important virulence factors.

This investigation has shown the presence of a novel haemolysin in *V. splendidus* and determination of the sequence of 8.7 kb of the *V. splendidus* genome has identified regions of homology with genes of *Vibrio* origin, with an almost identical gene arrangement to those in other *Vibrio* species (Figure 6.5). The only major difference between *V. splendidus* and other *Vibrio* sequences was the section of the genome that contained the haemolysin gene and this did not align with the DNA sequences of

other *Vibrio* spp. This sequence contains orfs with homology to the ToxR (Figure 6.6) and aerolysin genes (Figure 6.8) and it appeared that this had resulted from the insertion of foreign DNA into the *V. splendidus* genome.

6.4.1. Insertion of the aerolysin and ToxR genes

The presence of an aerolysin homologue suggested that there had been a recombination event in the *V. splendidus* DNA sequence but there were no discernible difference in the GC content of this region and other genes. However, as the splendilysin gene showed high homology with toxins produced by *Aeromonas* spp., which are members of the Vibrionaceae family, one might expect the GC content of *Vibrio* and *Aeromonas* spp. to be similar. An alternative analysis involved screening the sequence for inverted repeats using the EMBOSS Bioinformatics program. Several inverted repeats were identified before and after the splendilysin gene. The most promising of these was a 12 bp repeat found at the beginning and the end of the putative inserted DNA sequence. Insertion sequences are usually found flanking genes encoding traits such as resistance to antibiotics, resistance to heavy metals, toxin production, synthesis of amino acids and sugar utilisation (Kleckner, 1981). Therefore, these findings provide evidence that this section of DNA had undergone a genetic mobilisation event during the organism's evolution, perhaps through a transposon insertion or phage integration. The insertion in the *V. cholerae* genome (Figure 6.5) is further evidence that this area of the genome may be susceptible to DNA insertions.

There is evidence in other *Vibrio* spp. that such DNA mobilisation events have occurred. For example, the thermostable direct haemolysin of *V. parahaemolyticus* was found to have originated in another organism, with it being transferred as a transposon-like unit flanked by insertion sequence-like elements (ISVs/ISs) (Terai *et al.*, 1991; Nishibuchi and Kapur, 1995). However, transposition activity could not be demonstrated in the strain which may be due to the occurrence of base changes, insertions or deletions within the transposase gene during its evolution (Terai *et al.*, 1991). A similar hypothesis was proposed for the evolution of the alpha-haemolysin of *E. coli* (Zabala *et al.*, 1984).

Another example of a haemolysin that has become inserted into the genome of a *Vibrio* sp. is the haemolysin-conferring region of *V. pommerensis* (Jores *et al.*, 2003). This region was identified as having a low GC content and weak similarity with a *Xylella fastidiosa* transposase. Therefore, with the features of a genomic island, it was postulated that integration of foreign DNA had taken place. In addition to haemolysin genes, other virulence genes in *Vibrio* spp. have been found to be flanked by insertion sequences. These include the pJM1 genes that encode the proteins involved in iron-transport in the anguibactin iron uptake system of *V. anguillarum* (Tolmasky and Crosa, 1995; Di Lorenzo *et al.*, 2003), that has ISs highly related to those flanking the TDH genes (Terai *et al.*, 1991). Another example of a virulence gene flanked with ISs is the cholera toxin gene (Faruque *et al.*, 1998).

The fact that genes and Orfs are flanked by ISs does not immediately imply that the composite structures within them are transposons. Therefore, further work is required to identify the exact origin and the mechanism of DNA insertion that has occurred in the *V. splendidus* genome. This may prove difficult, as the transposition event may have occurred in the distant past and considerable evolution of the flanking elements may have occurred rendering identification of the insertion mechanism impossible.

6.4.2. Homology of splendilysin with aerolysin

Motile aeromonads are widely distributed in the aquatic environment and are the causative agents of haemorrhagic septicaemia of fish, reptiles and amphibians (Austin and Austin, 1999). *Aeromonas* spp. produce several extracellular toxins including haemolysins, enterotoxin, cytotoxin, acetylcholinesterase, phospholipid cholesterol acyltransferase and proteases (Cahill, 1990).

The high degree of amino acid homology of splendilysin with 12 aerolysin sequences is shown in Figure 6.8 and these alignments are plotted in a homology tree (Figure 6.10) which shows that the aerolysins cluster into two groups. Splendilysin appears quite different from these groups but has homology to each of the two clusters. Aerolysin is well-known as a pore-forming toxin and the osmotic protection studies identified these toxins as forming membrane pores of similar size. Therefore, it was assumed that by looking at the work on aerolysin, direct comparisons could be postulated between the two toxins. The pore-forming toxin aerolysin was identified

30 years ago by Bernheimer and Avigad (1974). Aerolysin produced by *A. hydrophila* forms heptameric pores in the membranes of cells. Estimates of the channel diameter can vary depending on the measuring procedure that is employed. Image analysis of the two-dimensional crystalline arrays formed by the oligomer produced pores with 1.7 nm diameter (Wilmsen *et al.*, 1992), while experiments measuring the release of small molecules from channels formed in the membrane of erythrocytes estimated the pore diameter at 3 nm (Howard and Buckley, 1982). Similar pore-sizes were observed for *A. sobria* aerolysin (Chakraborty *et al.*, 1990). Aerolysin toxin has been purified (Buckley *et al.*, 1981), the structural gene (*aerA*) has been cloned and sequenced from *A. hydrophila* (Howard *et al.*, 1987) and *A. trota* (Husslein *et al.*, 1988). Furthermore, the crystal structure of proaerolysin has also been solved (Parker *et al.*, 1994) and aerolysin monomers associate to form a heptameric membrane channel in the lipid bilayer. Other toxins that form heptameric pores include *Staphylococcus aureus* alpha-toxin (Gouaux, 1998) and anthrax toxin (Milne *et al.*, 1994). Numerous studies exist on aerolysin thus, making it one of the best-characterised bacterial toxins.

Aerolysin is synthesised as a preprotoxin containing an N-terminal signal sequence and a C-terminal activation peptide (Howard and Buckley, 1985 a, b). The signal peptide is required for translocation across the inner membrane into the periplasm where it is subsequently cleaved to form proaerolysin (Howard and Buckley, 1985a). This cleavage leads to the accumulation of toxin in the periplasm of the bacterial cells (Howard and Buckley, 1983). A later study concluded that the protoxin folds and dimerizes prior to being released from the cell, and correct folding is required for successful secretion to occur (Hardie *et al.*, 1995). This protoxin is secreted into the culture supernatant, where it is subsequently cleaved by proteases at the carboxy terminus into its active form (Howard and Buckley, 1985b). Cleavage of the aerolysin C-terminal peptide of about 40 amino acids exposes a hydrophobic patch (van der Goot *et al.*, 1992; 1994), but it is not known whether this patch is important in oligomerisation or in the insertion into the lipid bilayer of cells (van der Goot *et al.*, 1994). Proteolytic activation is also required prior to oligomerisation in other channel-forming toxins-the alpha-toxin of *Clostridium septicum* (Ballard *et al.*, 1993) and the haemolysin of *Pseudomonas aeruginosa* (Hayashi *et al.*, 1989). The cleavage sites within preproaerolysin are indicated in Figure 6.8, as deduced from the work of

Howard *et al.* (1987), but it is clear that the sequence of splendylisin is quite different suggesting that this toxin may be processed in a different manner.

Howard and Buckley (1982) found that murine erythrocytes contain a high affinity receptor for aerolysin and Gruber *et al.* (1994) identified this receptor as a 47 kD glycoprotein. However, the toxin also binds to other glycoproteins, including glycophorin of human and rat erythrocytes (Garland and Buckley, 1988). The glycoprotein receptor was further characterised and was found to be a novel protein that attached to the erythrocyte surface by a glycosylphosphatidylinositol (GPI) anchor (Parker *et al.*, 1996), that appears to be related to a group of similarly anchored enzymes involved in ADP-ribosylation reactions (Cowell *et al.*, 1997). This same group also identified Thy-1 as another surface protein that can act as an aerolysin receptor. Thy-1 is found in the brain and on the surface of T-lymphocytes and is also bound to the membrane through a GPI-anchor. Therefore, the GPI-anchor may be an important part of the aerolysin-binding determinant (Cowell *et al.*, 1997; Mackenzie *et al.*, 1999), which may aid in the lateral movement into the membrane.

After binding to erythrocytes the toxin must aggregate before being able to penetrate the lipid bilayer of the cells (Garland and Buckley, 1988) and the aerolysin receptor serves to concentrate the toxin on the cell surface, facilitating oligomerisation (Cowell *et al.*, 1997). The oligomerisation into heptamers leads to conversion of the protein from a soluble form into an insertion-competent form that penetrates the membrane and produces the channels that destroy the permeability barrier and cause cell lysis.

Based on the crystal structure of the proaerolysin dimer, each monomer is composed of a small globular lobe containing the first 83 residues (Parker *et al.*, 1994). Analysis of the two-dimensional crystalline arrays of the heptameric oligomer indicate that the large lobe forms an amphipathic β -barrel, that become the transmembrane channel (Parker *et al.*, 1994). It was found that both the small lobe and the region at the top of the large lobe of the toxin are involved in binding to GPI-anchors (Mackenzie *et al.*, 1999). This correlates with the fact that alpha-toxin of *C. septicum* binds to GPI-anchored proteins, despite it lacking a region homologous to the small lobe of aerolysin.

Other toxins that share homology with aerolysin have been identified; these include *S. aureus* alpha-toxin, *P. aeruginosa* cytotoxin, *C. perfringens* epsilon toxin, *C. septicum* alpha toxin, and perfringolysin O (Howard *et al.*, 1987; Ilayashi *et al.*, 1989; Ballard *et al.*, 1995; Parker *et al.*, 1996). These alignments are shown in Figure 6.14, modified from Parker *et al.* (1996), with the *V. splendidus* sequence included. This region also shows homology with alpha-toxin of *S. aureus* (Howard *et al.*, 1987). In addition, another toxin that has homologous regions to aerolysin is pertussis toxin (S2 and S3 subunits) (Rossjohn *et al.*, 1997); this is located in a different section of the sequence (not shown).

<i>A. hydrophila</i>	221	YDTATNWSKTNTYG-LSEKVT-TKNKEKWPL--VG	251
<i>A. salmonicida</i>	220	YDKATNWSKTDYIS-LSEKVT-TKNKEQWPL--VG	250
<i>A. trota</i>	221	YTMSNWSKTNTYG-LSEKVS-TKNKEKWPL--VG	251
<i>V. splendidus</i>	242	VDESTNWSKTNSYG-FSQSVQ-TENTFKWPL--VG	273
<i>S. aureus</i>	301	W-TSTNWKGTNTKDKWDRSS-ERYKIDW	312
<i>P. aeruginosa</i>	92	VDFTT-WSVTEQ---LKVGE-VKVKANIPL--VG	119
<i>C. perfringens</i>	107	CKNTDTVTATTTHT-VGTSIQ-ATAKETVPENETG	139
<i>C. septicum</i>	186	YTTSKTVSKTDNFK-FGEKIG-VKTSFKVGLAIA	218
Perfringolysin	181	YSESMVYSKQSQISSALNVNAKVLNSLGVDFNAVA	215
<i>S. aureus</i> alpha toxin	302	TSTNWKGTNT	311

Figure 6.14. Sequence alignment of homologous regions in a selection of channel-forming toxins. Amino acid residues with homology to *A. hydrophila* aerolysin are in bold text.

6.4.3. Homology with the ToxR gene

The homology with ToxR was not found in nucleotide comparisons, it was only recognised when the sequence was translated to amino acid residues. The amino acid sequence homology between the *V. cholerae* ToxR and the *V. splendidus* orf 3 was 29 % with only 159 nucleotides matching out of the 294 nucleotides of the *V. cholerae* ToxR gene. Therefore, the potential ToxR present in the *V. splendidus* genome is quite different as shown in the amino acid alignments with other ToxR genes (Figure 6.6). As a result of these nucleotide differences, the activity of the *V. splendidus* ToxR homolog may be quite different. However, the analysis of the structure identified that the ToxR homologue had a similar structure to the *V. cholerae* ToxR with the HTH motif and a transmembrane region (Figure 6.7). Therefore, the *V.*

splendidus ToxR may function to regulate the haemolysin in a similar manner to the regulation of the virulence factors by *V. cholerae* ToxR. The main difference is that the *V. splendidus* ToxR does not have the periplasmic domain. However, this may not affect the function of the regulator as a study by Crawford *et al.* (2003) found that this region was not required for transcriptional regulation of TcpP-dependent *toxT* or *ompU* and *ompT* genes. The study also found that ToxR did not have to be located in the membrane for transcriptional regulation of the *ompU* and *ompT*, however, it was required for the regulation of *toxT*. Therefore, mechanistic differences do exist in ToxR-mediated activation of promoters (Crawford *et al.*, 2003) and could explain the differences found in the ToxR of *V. splendidus*.

In general, successful infection by pathogenic bacteria is established by the coordinated expression of various virulence factors *in vivo* and this usually involves a global regulatory cascade or a two-component regulatory system (Miller *et al.*, 1989). These systems involve an environmental sensor which interacts with a transcriptional activator directly or via a transmitter. *Vibrio cholerae* has the *toxRS* system for this purpose, with the *toxR* and *toxS* genes clustered in an operon and encoding the transmembrane proteins ToxR and ToxS.

ToxR is a transmembrane transcriptional activator. The ancestral role of ToxR appears to be as a modulator of the expression of outer membrane porins, as it is able to activate directly and repress transcription of genes encoding porins OmpU and OmpT, respectively (Champion *et al.*, 1997; Miller and Mckalanos, 1988). The function of these Omps was discussed in chapter 4. The ToxR-dependent modulation of OmpU and OmpT in *V. cholerae* is critical for bile resistance, virulence factor expression and colonisation of an infant mouse model (Provenzano *et al.*, 2000; 2001). ToxR also regulates the expression of Omps in other *Vibrio* species (Kim *et al.*, 1999; Provenzano *et al.*, 2000).

ToxR of *V. cholerae* also indirectly regulates the expression of multiple virulence factors such as cholera toxin (*ctx*), toxin co-regulated pilus (*tcp*) and accessory colonisation factor (*acf*) genes by activating the transcription of the gene encoding a second regulatory protein, ToxT (DiRita *et al.*, 1991; Skorupski and Taylor, 1997a). Evidence from the transposon mutagenesis work on *V. splendidus* suggests that the

ToxR orf is involved in the expression of the haemolysin, as the transposon insertion in the ToxR gene in DMC-1-M2 (Figure 6.5) produced a haemolysin-negative phenotype.

A toxRS homolog was identified in *V. vulnificus* that had functional homology with the ToxRS of *V. cholerae* (Lee *et al.*, 2000). The ToxR in *V. vulnificus* was also found to control the production of haemolysin through the regulation of vvh gene expression. Lin *et al.* (1993) found that *V. parahaemolyticus* also has a toxRS homolog of the *V. cholerae* toxRS operon, which was involved in regulation of the expression of the thermostable direct haemolysin (TDH). Nevertheless, a ToxR from *V. anguillarum* serotype O2 was found to regulate the production of OMPs, but did not regulate virulence through the production of haemolysins or proteases, or in the resistance to bile, in an ayu fish model (Okuda *et al.*, 2001). In addition to this *V. cholerae* toxR mutants were found only to have a slight attenuation of virulence, indicating that ToxR is not a major regulator of virulence (Williams and Manning, 1991; Wang *et al.*, 2002). In addition, the ToxR mutants of *V. cholerae* had no effect on haemolysin production by the strain (Williams and Manning, 1991). Nevertheless, the presence of ToxR in other *Vibrio* species suggests that the toxRS operon may have played an important role in their survival throughout evolution with different virulence factors coming under the control of ToxR (Lin *et al.*, 1993; Lee *et al.*, 2000).

Another important point, with similarities in *V. splendidus* is the environmental factors that control the expression of the ToxR. The cAMP-co-regulated protein (CRP) was identified as influencing the expression of virulence genes regulated by ToxR (Skorupski and Taylor, 1997b). The cAMP-CRP system is known to function as a global regulatory network in enteric bacteria, such that in response to environmental carbon and energy sources the system controls the expression of a wide variety of genes through the nucleotide cAMP (Skorupski and Taylor, 1997a). The involvement of cAMP-CRP in the regulation of cholera toxin and tcp expression in *V. cholerae* allows an understanding of the mechanism by which environmental stimuli control the expression of ToxR. The mechanism involved is catabolite repression, that occurs as a result of a rapidly metabolisable carbon source, such as glucose, being added to the growth medium which leads to repression of the synthesis of many

enzymes required to metabolise other carbon sources. Therefore, the addition of such a carbon source to *V. cholerae* cultures would repress the ToxR regulon (Skorupski and Taylor, 1997b). This would explain why the expression of the ToxR regulon may be favoured in the nutrient-rich environment of the stomach with the intestinal glucose levels influencing the colonisation of the pathogen through the control of virulence factor expression (Skorupski and Taylor, 1997b).

Therefore, this effect may also explain the reduction of splendilysin production in *V. splendidus* when glucose was added to the culture medium, (Appendix 10). This effect is shown in other bacteria, for example the expression of certain pili in enterotoxinogenic *E. coli* strains is negatively regulated by glucose (Isaacson, 1980). The result of this is that the pathogen efficiently colonises the distal regions of the small intestine, where glucose levels are lower than in the more proximal areas (Ferraris *et al.*, 1990). The addition of glucose caused the same effect as that discussed above, in haemolysin production in *V. vulnificus* (Bang *et al.*, 1999). In this case it was found that haemolysin expression was dependent on cAMP and *crp* gene function. Therefore, overall the ToxR gene located upstream of the splendilysin gene may be under similar regulatory control to those discussed above.

However, catabolite repression could be just one of the mechanisms involved in the regulation of ToxR of *V. splendidus* to control the haemolysin expression, as bacterial pathogens usually have highly sophisticated systems involved in the regulation of gene expression to enable efficient colonisation of the host. For example, an additional regulatory gene in *V. cholerae* involved in the expression of El Tor haemolysin (HlyA) was originally identified as hlyR (von Mechow *et al.*, 1985), which was found to be distant from HlyA, but closely linked to *toxR*. The regulator of this haemolysin was later re-identified as hlyU in a novel locus (Williams and Manning, 1991). Therefore, the ToxR homolog in *V. splendidus* may be a novel regulator acquired during evolution to regulate the novel haemolysin gene for this particular *Vibrio* species.

Chapter 7. General Discussion

In this study *V. splendidus* strains from a study by Thomson (2001) were employed to identify the virulence mechanisms involved in the pathogenesis of this bacterium. The virulence of *V. splendidus* biovar 1 strain DMC-1 was confirmed in turbot larval challenge trials. Earlier studies by Gatesoupe *et al.* (1999) identified *V. splendidus* biovar 1 as a turbot larval pathogen.

Vibrio splendidus DMC-1 was pathogenic towards first-feeding but not towards yolk-sac turbot larvae. Therefore, this provided evidence that the bacterium needs to be delivered into the gut before any pathogenic effects occur. This is not the case for the marine pathogen *V. anguillarum*, which is known to be invasive (O'Toole *et al.*, 1996) and can cause mortalities in the yolk-sac larval stages (Hjelm *et al.*, 2003). The immunohistochemistry sections clearly show that the *V. splendidus* DMC-1 strain caused damage in the gut with enteritis visible. The same work with the haemolysin negative transposon mutant, DMC-1-M2 found that the bacteria adhered to the gut epithelium with no signs of enteritis, showing that the this damage is a result of the action of splendilysin.

On further characterisation of splendilysin using molecular techniques it was found that it had homology with aerolysin. This is the first discovery of an aerolysin homologue outside an *Aeromonas* spp. Aerolysin is one of the best characterised pore-forming toxins, thus, allowing good comparisons to be made with splendilysin. In fact the homology with aerolysin provides further evidence that the splendilysin is responsible for the enteritis in the larval gut. In addition to this, the large protein bands (>200 kD) identified in ion-exchange column chromatography fractions of partially purified splendilysin could be aggregates of active toxin (identified from the amino acid sequence as having a MW of 55 kD), as the related aerolysin (a 52 kDa enterotoxin) is known to form dimers in aqueous solution.

The alignments of the *V. splendidus* genomic sequence with the three *Vibrio* spp. gene sequences were almost identical apart from the section of the *V. splendidus* genome that contained the orfs VS2 and VS3. The VS2 gene with homology to a ToxR transcription regulator must have some control over the expression of the splendilysin

as the transposon insertion in this gene in the DMC-1-M2 mutant strain produced a haemolysin-negative phenotype.

The addition of glucose to the growth medium inhibits haemolysin production, by a possible catabolite repression mechanism, which may be related to the ToxR homologue. Previous studies on ToxR in *V. cholerae* have shown that it is controlled by the cAMP-CRP, which is down-regulated by the addition of glucose (Skorupski and Taylor, 1997b). This was also shown in the control of expression of the *V. vulnificus* haemolysin, where the addition of glucose repressed production and the addition of cAMP enhanced production (Bang *et al.*, 1999). Therefore, the addition of glucose to the growth medium may mimic the conditions that occur in the gut after the cells have been damaged by the toxin; the gut cells will release glucose, which in turn will switch off toxin production via the control of the ToxR homologue. This action would prevent the toxin damaging the bacterial cell integrity. Therefore, it is predicted that the ToxR acts as an environmental sensor that controls the virulence mechanism of toxin production enabling the bacteria to survive within the host.

It was speculated in chapter 6, that the section of genome in *V. splendidus* containing the toxin regulon occurred as a result of a DNA mobilisation event, either via transposon or phage integration, with IS sequences identified. Pathogenic clones of *V. cholerae* evolved from aquatic forms and attained the ability to colonise the human intestine by the progressive acquisition of genetic information (Faruque and Mekalanos, 2003). The main virulence factors of *V. cholerae* are encoded on mobile genetic elements that have been acquired via phages or horizontal gene transfer (Faruque and Mekalanos, 2003) and are clustered on the chromosomal DNA with the donors of these gene clusters remaining unidentified. The gene alignments (Chapter 6, Figure 6.5) show the insertion of the toxin within the *V. splendidus* genome; also highlighted is the large insertion in the *V. cholerae* genome, therefore, this area of the *Vibrio* genome may be susceptible to DNA insertion. The exact origin of the splendilysin is not clear as shown in the consensus tree (Figure 6.8). The toxin is quite different from the two clusters of aerolysins but does have homology with each. However, no aerolysin is particularly closely related to splendilysin.

The ToxR homolog associated with the splendilysin gene had no matching genes in the *Aeromonas* spp., therefore, *V. splendidus* may have acquired this section of the genome from another bacterial species, for example another *Vibrio* spp. As shown in chapter 6, the ToxR gene in *V. splendidus* had structural similarity with ToxR of *V. cholerae* identifying a HTH structure (Figure 6.7). Therefore, as discussed in chapter 6 the *V. splendidus* ToxR may act in a similar manner to the *V. cholerae* ToxR. Over time, the latter part of the *V. splendidus* sequence may have undergone various mutations leading to a quite divergent sequence, but this may not affect the action of ToxR as a study by Crawford *et al.* (2003) showed that membrane localisation and the periplasmic domain were not a strict requirement for DNA binding and transcription activation by ToxR. The divergence of the sequence over time makes it difficult to assess the exact origins of this toxin gene regulator. This ToxR regulator could be present in *V. splendidus* to control the haemolysin toxin which may act completely separate from a conventional ToxRS system which may also exist in *V. splendidus* strains.

Other virulence factors identified as being associated with this strain include the presence of the dominant Omp that has homology with OmpU of *V. cholerae*. As discussed in chapter 4, this Omp may play an important role in the survival in the gut of the host. It may be acting as an adhesin and may be responsible for the haemagglutination activity of the turbot erythrocytes. It might also be involved in bile resistant activity in the gut as shown in other *Vibrio* spp. (Provenzano *et al.*, 2000; Wang *et al.*, 2003). Both of these actions would enable the bacteria to survive and colonise the harsh environment of the gut. The OmpU of *V. anguillarum* has been shown to affect bile resistance and biofilm formation. Both of these activities aid the survival and adaptation of the bacterium in seawater and in the fish host (Wang *et al.*, 2003). The Omp may have been dominant in the ECP of *V. splendidus* as a result of the growth medium being a rich source of nutrients that favours growth, thus mimicking the conditions in the gut. Therefore, if this Omp is produced in a similar manner to the OmpU of *V. cholerae* then it would be up-regulated in these nutrient rich environments to aid survival. It is possible that another Omp is present that is down-regulated and is stimulated in nutrient-limited conditions as shown with the OmpU and OmpT of *V. cholerae* (Wibbenmeyer *et al.*, 2002). The appearance of two proteins, one of which was identified as an Omp, in the different growth media may

have been evidence of this effect, with proteins at 36 and 38 kD (OmpU) being produced by MB and TSB + NaCl, respectively. The latter growth medium has a higher glucose content, which did reduce the production of splendilysin. This catabolite repression may be responsible for the altered production of the Omps of *V. splendidus* as altered expression of Omps has been found in *V. cholerae* (Provenzano and Klose, 2000).

Another possible virulence factor was the weak protease that may be related to the activation of the haemolytic toxin. This would apply, if as with the aerolysin, the splendilysin is secreted from the bacterial cell as a protoxin, that is subsequently activated to its active form by proteolytic cleavage at the carboxy terminus (Howard and Buckley, 1985a). The activation of proaerolysin enables the bacteria to be protected from the pore-forming toxin before being released from the periplasm. The activation site in aerolysin is a highly mobile region that is not resolved in the crystal structure, but is known to be located at the tip of the large lobe of the toxin in domain 4. This region contains sites for a variety of proteases, including trypsin, chymotrypsin and furin (Garland and Buckley, 1988). Therefore, the presence of proteases in the gut makes it an ideal environment for the activation of the protoxin. In addition to the reliance on host proteases, the *Aeromonas* bacteria secrete at least one protease capable of activating aerolysin (Garland and Buckley, 1988). Therefore, the protease may be produced in *V. splendidus* for the same reason.

7.1 Future work

Firstly, the haemolysin gene should be complemented back into the mutant strain, DMC-1-M4 in order to see if virulence is returned to the bacterial strain. As the strain contains the ToxR, there is no need to complement this gene back. However, a second complementation could be carried out with DMC-1-M2 and the ToxR gene. Complementation of the gene back into the mutant strains would allow them to be tested for haemolytic/cytotoxic activity in both the erythrocyte and the turbot tissue culture cell assay, which could be followed by the confirmation of the activity *in vivo*, in turbot larval trials. This would prove that the haemolytic toxin was responsible for one of the virulence mechanisms of this strain, alongside others in a multifactorial action.

In addition, further studies are required on the adhesion mechanisms of this strain. A turbot-specific adhesin was recognised in the agglutination assays with the turbot erythrocytes. With the knowledge of the homology to aerolysin, the application of aerolysin antibody to either erythrocytes or tissue culture cells would assess if the toxins had similar receptors. The immunohistochemistry studies with the haemolysin negative mutant DMC-1-M2 challenged turbot larvae showed that the bacteria adhered to the gut but did not cause enteritis. The haemolysin-negative mutant strains could therefore be used as model strains for studying the adhesion mechanisms in *in vitro* assays with turbot tissue culture cells TV1-S4.

On the whole it is necessary to obtain purified toxin in order to carry out a more detailed study on the action of the toxin as well as identifying the structure of the toxin. By carrying out this work it would provide more data for further comparisons with existing aerolysins.

In addition to the work with the toxin, further work with the larval rearing systems would be advantageous. The identification of virulence mechanisms of pathogens aids in the search for potential probionts. The haemolysin-negative mutants were able to colonise the gut and prevent the colonisation of the parent pathogenic strain, DMC-1 without any damaging effects, thus, showing that these mutants have qualities of a

potential probiont. However, as these strains were genetically modified they would not be acceptable for use in the flatfish rearing systems. Nevertheless, a group of *V. splendidus* biovar 1 strains (LMS-1 to 4) with a haemolysin-negative phenotype confirmed by PCR (Chapter 6) were identified from a larval rearing system (Thomson, 2001). Therefore, these strains would be more acceptable and have the potential to be tested *in vivo*. This would involve a challenge trial with LMS strains and the DMC-1 strain against turbot first-feeding larvae to assess if competitive exclusion occurs. It is postulated that these strains would be able to colonise the gut in the same manner as the transposon mutants and cause no pathogenic effects. As the LMS strains are *V. splendidus* biovar 1 they would potentially bind to the same receptor sites as DMC-1 and block the pathogen from colonisation and thus prevent virulence.

Nevertheless, it is worth bearing in mind that the haemolysin gene was proposed to have been acquired in the *V. splendidus* genome by means of a DNA mobilisation event. Therefore, the LMS strains could have potentially lost this genetic element in the rearing system, which may mean that they would be susceptible to future 'cross-over' events of this toxic element from pathogenic strains. Therefore, it would be desirable to identify the exact mechanism by which the *V. splendidus* biovar 1 strains have acquired this genome insertion in order to fully understand the pathogenesis of *V. splendidus* strains.

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Appendix 1

Bacterial Strain	Identified ¹	Haemocyte Rounding ²	Bacterial Strain	Identified	Haemocyte Rounding	Bacterial Strain	Identified	Haemocyte Rounding
DTC-5	<i>V. splendens</i> bv. 1	High	S3L1-8	<i>V. splendens</i> bv. 1	Low	S1A1-4	<i>V. alginolyticus</i>	Low
LTS-3	<i>V. splendens</i> bv. 1	High	S3L1-9	<i>V. splendens</i> bv. 1	Low	S1A1-5	<i>V. alginolyticus</i>	Low
S1L1-2	<i>V. splendens</i> bv. 1	High	S3L1-10	<i>V. splendens</i> bv. 1	Low	S1A1-6	<i>V. alginolyticus</i>	Low
S1L1-3	<i>V. splendens</i> bv. 1	High	S3L1-11	<i>V. splendens</i> bv. 1	Low	S1A1-7	<i>V. alginolyticus</i>	Low
DMC-1	<i>V. splendens</i> bv. 1	Medium	HNF-8	<i>V. splendens</i> bv. 1	Low	S1A1-8	<i>V. alginolyticus</i>	Low
DMC-3	<i>V. splendens</i> bv. 1	Medium	HNF-2	<i>V. splendens</i> bv. 2	High	S1A1-9	<i>V. alginolyticus</i>	Low
DMC-4	<i>V. splendens</i> bv. 1	Medium	HNF-5	<i>V. splendens</i> bv. 2	Medium	S1A1-10	<i>V. alginolyticus</i>	Low
DTC-1	<i>V. splendens</i> bv. 1	Medium	LTH-1	<i>V. splendens</i> bv. 2	Medium	S1A1-11	<i>V. alginolyticus</i>	Low
DTC-2	<i>V. splendens</i> bv. 1	Medium	LTH-2	<i>V. splendens</i> bv. 2	Medium	S1A2-2	<i>V. alginolyticus</i>	Low
DTC-3	<i>V. splendens</i> bv. 1	Medium	LTH-5	<i>V. splendens</i> bv. 2	Medium	S1A2-4	<i>V. alginolyticus</i>	Low
DTC-4	<i>V. splendens</i> bv. 1	Medium	HNF-5	<i>V. splendens</i> bv. 2	Medium	S2L1-2	<i>V. alginolyticus</i>	Low
DTR-1	<i>V. splendens</i> bv. 1	Medium	HNF-3	<i>V. splendens</i> bv. 2	Low	S2L1-3	<i>V. alginolyticus</i>	Low
DTR-3	<i>V. splendens</i> bv. 1	Medium	LTH-3	<i>V. splendens</i> bv. 2	Low	S2L1-4	<i>V. alginolyticus</i>	Low
DTR-4	<i>V. splendens</i> bv. 1	Medium	LTH-4	<i>V. splendens</i> bv. 2	Low	S2L1-6	<i>V. alginolyticus</i>	Low
DTR-5	<i>V. splendens</i> bv. 1	Medium	LTH-1	<i>V. splendens</i> bv. 2	Low	S2L1-7	<i>V. alginolyticus</i>	Low
DTR-6	<i>V. splendens</i> bv. 1	Medium	HNF-3	<i>V. splendens</i> bv. 2	Low	S2L1-8	<i>V. alginolyticus</i>	Low
DTR-7	<i>V. splendens</i> bv. 1	Medium	HNF-4	<i>V. splendens</i> bv. 2	Low	S3A2-2	<i>V. alginolyticus</i>	Low
DTR-8	<i>V. splendens</i> bv. 1	Medium	HNF-6	<i>V. splendens</i> bv. 2	Low	S3A2-3	<i>V. alginolyticus</i>	Low
S1L1-4	<i>V. splendens</i> bv. 1	Medium	HNF-7	<i>V. splendens</i> bv. 2	Low	S3A2-5	<i>V. alginolyticus</i>	Low
S1L1-5	<i>V. splendens</i> bv. 1	Medium	HNF-8	<i>V. splendens</i> bv. 2	Low	S3A2-9	<i>V. alginolyticus</i>	Low
DMC-2	<i>V. splendens</i> bv. 1	Low	S3A2-6	<i>V. alginolyticus</i>	High	S3A2-10	<i>V. alginolyticus</i>	Low
DMC-5	<i>V. splendens</i> bv. 1	Low	S3A2-7	<i>V. alginolyticus</i>	High	S3A2-14	<i>V. alginolyticus</i>	Low
DMC-6	<i>V. splendens</i> bv. 1	Low	S3A2-8	<i>V. alginolyticus</i>	High	S3A2-19	<i>V. alginolyticus</i>	Low
DMC-7	<i>V. splendens</i> bv. 1	Low	S3A2-15	<i>V. alginolyticus</i>	High	S3A2-20	<i>V. alginolyticus</i>	Low
LTS-1	<i>V. splendens</i> bv. 1	Low	S3A2-22	<i>V. alginolyticus</i>	High	S3A2-21	<i>V. alginolyticus</i>	Low
LTS-2	<i>V. splendens</i> bv. 1	Low	S1A2-1	<i>V. alginolyticus</i>	Medium	S3A2-23	<i>V. alginolyticus</i>	Low
LTS-4	<i>V. splendens</i> bv. 1	Low	S1A2-3	<i>V. alginolyticus</i>	Medium	S3A2-24	<i>V. alginolyticus</i>	Low
LTS-5	<i>V. splendens</i> bv. 1	Low	S1A2-5	<i>V. alginolyticus</i>	Medium	S3A2-25	<i>V. alginolyticus</i>	Low
DTR-2	<i>V. splendens</i> bv. 1	Low	S2L1-1	<i>V. alginolyticus</i>	Medium	S3A2-26	<i>V. alginolyticus</i>	Low
DTR-4	<i>V. splendens</i> bv. 1	Low	S3A2-1	<i>V. alginolyticus</i>	Medium	S3A2-27	<i>V. alginolyticus</i>	Low
LMS-1	<i>V. splendens</i> bv. 1	Low	S3A2-4	<i>V. alginolyticus</i>	Medium	S3A2-28	<i>V. alginolyticus</i>	Low
LMS-2	<i>V. splendens</i> bv. 1	Low	S3A2-4	<i>V. alginolyticus</i>	Medium	S3A2-29	<i>V. alginolyticus</i>	Low
LMS-3	<i>V. splendens</i> bv. 1	Low	S3A2-11	<i>V. alginolyticus</i>	Medium	S3A2-30	<i>V. alginolyticus</i>	Low
S3L1-1	<i>V. splendens</i> bv. 1	Low	S3A2-12	<i>V. alginolyticus</i>	Medium	S3A2-31	<i>V. alginolyticus</i>	Low
S3L1-2	<i>V. splendens</i> bv. 1	Low	S3A2-13	<i>V. alginolyticus</i>	Medium	S3A2-18	<i>V. pelagius</i>	Low
S3L1-3	<i>V. splendens</i> bv. 1	Low	S3A2-16	<i>V. alginolyticus</i>	Medium	HNF-2	<i>Marmomonas vaga</i>	Medium
S3L1-4	<i>V. splendens</i> bv. 1	Low	S3A2-17	<i>V. alginolyticus</i>	Medium	HNF-7	<i>M. vaga</i>	Medium
S3L1-5	<i>V. splendens</i> bv. 1	Low	S1A1-2	<i>V. alginolyticus</i>	Low			
S3L1-7	<i>V. splendens</i> bv. 1	Low	S1A1-3	<i>V. alginolyticus</i>	Low			

¹ 16S rRNA gene sequencing results (Thomson, 2001); ² Haemocyte cytotoxicity assay results.

Appendix 2

Primer list

cat forward
GCG TGT TAC GGT GAA AAC CT
cat reverse
ATC ACA AAC GGC ATG ATG AA

Tn10D
CCT CGA GCA AGA CGT TTC CCG
Tn10C
GCT GAC TTG ACG GCA CGG CG

internal cat
TGC TCA TCG AAA ACG GTG TA
inverse cat reverse
TTC ATC ATG CCG TTT GTG AT

M2-DMC-1 Forward 1
TTG TTC ACA ATC TCG GGT GA
M2-DMC-1 Reverse 1
AGC GTT TGC TTT TGT TCG TT

M2-DMC-1 Forward 2
AGG TTT GGA ATT GCC ATC AG
M2-DMC-1 Reverse 2
CAC AAC CCC TTT TTG CAG TT

M2 Forward 3
AGC GAT GCC TGA TTG GAT AG
M2 Reverse 3
AGC AAG CTT AGT GCC CGT TA

Tn cat left
GCC GAT CAA CGT CTC ATT TT

M4 internal 1 forward
AAC GGT TTG AGC CTC ATC AA
M4 internal 1 reverse
CCG CTA CAA GAA CCA CCA TT

M4 Internal 2 forward
TTG TAG CGG TTA TCG ATG TG
M4 Internal 2 reverse
TTG TGC CAT GCA GTT AAC AAG

M4 Internal 3 forward
TGT GAC GGA AGA TCA CTT CG

M4 Internal 4 forward
TTT TTC GTC TCA GCC AAT CC

DMC-1 hly1 Forward
CAA CTC GAA TCG GAA GCT CT
DMC-1 hly1 Reverse
AGC CGA AGA GCA AAA GAG TC

M4 TOPO A1 forward
CTG CAG ACA TTC GCT ACC AA
M4 TOPO A1 Reverse
GTT AAG TTA GCG GCG TCT GC

M4 TOPO A2 forward
CGA GTT CAA TGC ACC AGC TA
M4 TOPO A2 forward
CCC ATA AGG CTG CAT CAG TT

Vsplen hly reverse
GGC AAT CTG GTT TAG CTG GT
M2/M4 forward
TGC GTT GTG TTC TTG CTA GG

M2/M4 forward 2
CAT CGC ATT AGT GGC TTC TG
M2/M4 reverse 2
ATG TCC GAA TGA TGG CAA AC
M2/M4 forward 3

GCC ACG TGG TTA TCG AAG AA
M2/M4 reverse 3
CAA CAC TGC GGT TGC TAA GA

M2/M4 1.0k Forward
TTG TTT GAA CCA CGC AAA AA
M2/M4 1.0k Reverse
GAT GTA TGC CAC GCA ATC AC
M2/M4 1.5k Forward
CCA AAA CGG CGA CAA AAT AT
M2/M4 1.5K Reverse
AAA AGC TCC CTA ATA AAA TCA AAA

Appendix 3

1. Haemolytic clones from plasmid genomic library.

HD-1 Primer M13 forward

GTATTAGCCGCGACATGGGCTTGGTGAAGAAATTAACCAAGTTCACATCATTTGGTGGTGTGATGGTGATTTCAGGCGCTCATCCTTGC
TCAAAATCAAAAGAAAGCCAGGCTTGAGGCTCCTCTCCCTCAGCAAAGCTAAAGCAATGGGACAAAGCTCATATTAGGGCAACAC
TCAAACTCACTTTTTCACAGTGGCCGACTCCTAAAGTAGGCAGCTGTGTTTATTTACAGTCTTTCAAATGCTTTTAATAGGCTT
CATTGTGGTTGCAACATGTTAAGGAGTCAAGGTTTAAATGCATTAGTACCGTTTAAAGTGTAAAGGAATTACAACTCACTCAACCAA
CGATCCCTGTGATCATCTCGCTCCCTGCTTGTCTTTATCATCTGCTTCAAGCTTTGCTGCCACTGTTCCTGCTGTATACACCATTAGCT
GAGGAACACATTTTGTACGTGGCAACGCTGCAGAGCCCAATACACTAGATCCTAGCTTCGTAAGTCAAGGCATGCCCGGTGACAT
TATCGTCAATGATATCTTTGAAGGCTTTGTGATTGAGAACAGTGACGGGCAATCATCCAGGSCAAGCCAAAGAGTGGTCTATCA
GCAAAGACGGTAAACCGTACGCTTGTATGAAAGAGAACCTCAAGTGGTCAATGGGAGCCAGCTACAAGCGTCTGATTTTGT
GTTTGGGTGGCA = 700 BASES

HD-1 Primer M13 reverse

AGTTGGGAAAAAGGTCAGCTAGGACCAATGATTAGTTAGGGAATTAATTAGCTAGCTACTAGGAACGATTAGCGATGAAGTGTAG
AAGCTAGAATCTTTTGTCTCGAAGGAGCACAGCCCAAGTGTGCAACACACATAGATGCTTTCTTGATCGAGTCCACACGGCGAA
AGAGATCCGCAACGCTCTTATCCCGCCAAACAAAGTCTGGATATAGTGGTAAATTTCAATTGCTATCGTAACCTTCTACGTACAAT
GTTCTGACCCATTGGTACTCAACTGCTTGGATTGTTCAAGTAGATTCGCTAAGAGTGGGGCGTGTGACGCTCAAGAGTAGCTC
CGGGAAATTCAAATACATCCATTATGATTGGCCGTATTTAACCAGAGCTAAATGACGACTTTATTACAGTCCGAGTAATCGATAA
ACAATACCGGCTTCAATTCACCCCTTGGCTGAGTATCACGCTATATAAGCTGTATTGAGATAAGTAATATTGAGATAAGCAGTATT
GGATAAGACCGGCTTAGCCTGTGAGAGTTTGTATAGGGCAGTTGAGTTAGATAAGTAATGCCAAGCATGTGCTTGGCATTGTAA
CTAGGTTTGTAGGTAGGGCTAGATATGGACCTTATTTAA = 643 BASES

HD-2 Primer M13 forward

ATCAAAGCATCGCAAACTGGTCACTTAGTCTTTCGACACTGCACACTAACTCTGCAGCGGAAACCGTCACTCGACTCGCTCATAT
GGGGATAGAACCCCTTAACCTAGCTTTCATCACTGAGTTTGTATTATCGCTCAACGACTAGCAAGACGATTGTGCAACCATTTGCAAAG
AAGTTGACGACTCACCGGACATATTTCTGCTCACTCGATTCTTAACAGCCGAACCATCTACACGCTAATCCCAAGGGTGTAAAT
GATGTAAATCAGGSACTACTCAGGCCGAGTGGGTATCTATGAAGTGAATGCTTTTACCAGACAAGCTCAAGACGAGCTTATCACTCA
ACCAATGCGGTAGCGATTGAAGATCTCGCGCTCGAGAAGGCATGAGAATCTGCAAGAGTCAGGGCTAGATAAATGCTTTGAAG
GCACCCACGATATCAAGAAGTCAACGCTTCTGTACCTATACCGAATGGAGCACCAATGAGTAGCAAGTCCAAACAATCACAA
TTAAAGAGCTATCACTGGAAGAGGCTAATAGCTCAGGCAAGAAAGTGTCTGGGAGAGCTTAGCGCTCACCGAATTTGGAAGTACG
AGAAAGCTCAAAGAGCAGTACATTACAGTTAAGAAAATCAAAAGAAAGCATCTCAGCCGCTCACTTCTGTTTAAACCATAGAGTC
AAAG = 693 BASES

HD-2 Primer M13 reverse

AAAGATGGAACGAAAGAGTTGATACCTGTTGAGCATCGGAAATGTAGAAGACAAGTGTAGTGGCTCAAGAGTGTATCGACTC
TTAAGTAGATCGCTGTTTCTCATTTAGAGGATGTTCAAAATTTATGGGTGATCATCGAGCAACCTTCAATCAACATTGTCTATPT
CTGACCCATCTACAGCACTGATTGCCAGATACTTTTGTATTAATCTGTGATTTGAGGCAAAAGTCTCTGGTTTGTAGTATGGTTT
TTAATCAGCTCATCTGCAACTGCTAAGGCTTGTCTCTGGAAGCTGTGATTTTAAATTTGATGCAACTTGTTCCTTAGAAGATT
ATCAGCACTCATCGTTCGTTCTATTGCACTTCTGCTGGCACATCAACGATTAAATACGCCATCGGTCAATGCTTCATTTGGTTTT
CGACCAATAGCGGTGCGACTAATAAACCATAGGGGATGTGATTTTAGACAGGTCAGTGTCAATTTTGTCAAGCATCATGGGATGA
AGAAGATCATTTGAGCCACTGCTTCTCTTTAGGATCAGAGAAGATAAGCTCACGCAAGCTACTTCGGTTGAGTGTCCATCTGTGAAG
CAAGATAGACTCAACAAAGTGGTCTGTATCTGTTTAAAGCTCACTGGCCRAAGCGACTACTTCACCGTGGCCAGATATCAGCA
TCAACCATATCAATATAAGGTGTTTATTGGCAAGTTAGCGAC = 733 BASES

HD-3 Primer M13 forward

CTGTTGAAATGCCAGCTTGTGCGGTGGCTTACTTCGACCGCTTTGCTGCACAGCCTAGGCGTTGCAAGGGCAAAACAACACTA
CTGAAATGGTCTCTCAGTCTTGCTTTTATTACGTTTGTGTTGAGTATCTTAGGCACCTTTATTTGTTCTGCTTGGCGTACTTACATC
GGTACCGCTTTTGTCTTTGACCCAAACCAAGGATCGCACTGCTGCTTATATTGGTTTGGTACTGGTGAGCTCATTTCTCTGCT
TTATCGTTAGAGGGGAGTCTTTGAATCAAGCCCTATTACCAGCTTCGCCAGTAAGAGCTTTTGAGTTTASTCGCGGTACTCATTT
TTTGTCTCGCAACAGCCGTTGCTGTTTTCGGCACATTTTATCCAAATGGTATTTGAAGTGTCTGGAGTGGGTAACATATCGGTAGG
TCCCGCTTACTTTAATCTATTGATTCGAACCTTTGGCGTTGCTTGCCTTGGCCGCTGTAGGTTTACGCGCTTTGTTGAGTATTAAAGC
CTCAAGCGTCAAGAGCGCTTATTATGTTGATAAGCTCTAGCATCTATTGTCTA = 570 BASES

HD-3 Primer M13 reverse

AGCCGCTCTCTGGTTAAGGCTATCAATGCCAATCACACCAGCAGCAGCTCAATCGCAGCCGTTTGAGACACTAGCGCAACAAATG
TTTTGTTTACTAGTGGTTACCCGAAGCGTTGTGACAGCAATTAGCTGATCGAAATGCTGGTTGTTTTCTCTGCAGAAACACCA
AGCTGGCCAACTTTGTGACGCTGCTAAGTCAAACTGCTTTTGGATGCTCTTCGCAAGCTTGTCTCATCTTTTGCTCTACTAGGTCAAA
CAGGCTAGGACCTGAAAGCAGAGTACCGTCTTCAGGCTGTATAGACCGGTATAAACGTTGTAGATACCTTGCTCGTTGTAGTAGT
GAGAGTGTGCGGTGTATCAGAGAAGCAATCGTGTCTATCTTCAGTAGAGTTAGCTTCTAAAGCCACCTTCATACGCTCACCAGCA
AGCTCACCAAGAGACAGTGAACCCATGCCGAATAACATTTTACGTAGGCGGTTTTCGCTAGATTGAGAAAGCAGTCTCTTGACGGTA
GTTACCTTTTTCGCTTCAGCCCACTGCTTTTCCATCCACTCTAGGTCCT = 566 BASES

HD-4 Primer M13 reverse

GGCGCTGTGTAGTCAGGAATATTATTCGGGCTCACGCTGTATGCTTCTGGTTCACTTGAACCTTCTCGACAGAAATCTC
ACGATTGACGCGCATGCTCAGTGTGCGCTACTCTTACGTCATCAAAACGGTGTGCTGACGTGTATTGAATGAATAAACGTAAGAGC
CAATAGAGCTTGGCTTGTGATTGCTCAGGCTTTCTTGGATTAAATTTTGGTACTACTCAAGCTGGACACCATTTGGTCAATATCA
ATTTCAACAGCTTGGTAACGAATCAGCTCAGCATTTTGAGAGGATAAGGCTAAATAGGTCACCTTGTGTAGTACTGTGACGCTGTT
ATCCCAATAAATTTGGGTTTCTCTCGCTTCAAGGATTCGTTGGGTACCCATTTACTCAAAGTGTAAAGCACCGTTGGTCCGATAT
TTTCTGCGGAGTCCATTGGTGGCTTTCTCTTGAACCAACTTASCGGGTAGAGGGAAGAGCTCTGTATGCTCATTAACCTCAT
AAGTAGGCGGTAGCTTTTGAAGTGAATTTCAACCGTGGCTGTATCTAATGCTTTGATGCCAAGCTCAAGAGGAGC = 593
BASES

HD-8 Primer M13 forward

AGCTCGGCTTGGAGTCTCGGCTACCTTAGCAACAGTCAGAAGTATTCGGCTTCGGCAACGCTGTGTGATGATTGGGAAGGCCAAC
TGAACGCATGGCCACTAGATGAAGGCGCTGATTGACTACGTATCTTCTGATTACCAATATGAGCTTGGTAACGAAGGIGCTAGCGCA
AACATCGTGGCTAACAGACTTTCCAAATTGGTCAGACTACGTTGATGCAACTAACATTACTCCAGAGCTAATCGCTGACTTGAA
CGAGATCGGGGCTTCTGAAGGCAACGCTAGCGCTCGGCTACCCACGCAATTGAATTCCTACTTTGGGGCCAAAGATTAAACGGCACAA
ACAGCGGTGCAGGTGAGCGTGCTTACACTGATTCGTTGTTGGTGCTGAGTGTACTAACGGCAACTGTGACCGTCTGGCGCATAT
CTAAAGCATCTGCTGAGCTACTTATCCAGAGCCTAGAGTGGATGGAAAGCAGTGGGCTGAAGCGGAAAAGGTAACCTACCGTCA
AGAACGCTTCTGTGATCTAGCGAAAACGGCCTACGTAAATGTTATTCGGCATGGGTTCAGTGTCTCTTGGTGAGCTTGGTGGT
AGCGTATGAAGGTGGCTTTAGAAGCTAACTCTACTGAAGTGAAGCAGTATGCTTCTCTGATAACACGCCAACT = 677
BASES

HD-9 Primer M13 reverse

CTCTAGAGTCGACCTTGAAAAAATTGTTAACAGCCTCATAGTGTCTATCATTAGATAGTTAGCTACAAAATATTGCCTAGACATA
TAATGTAGTGTGCTTAAACACATCGAATTTCAAGTGAATATAGCTATGGCAGAGTGCCTGCCAGAAATAGATTTCAAAGTAGGGG
TAACAACCAATTGATGCTGAACCTCTGCTTTTCATGGATTGAAAACAGACAAAGAGCATGTTGCTGTGATATTTAAATCAGCA
GATAAGACACAAGACATACCTCTAGTTCGTATGCACTCTGAGTGCTTACTGGAGATGTTTCCATTCTCTCGCTGTGACTGTGG
CGAGCAACTAGACGAACCAATTAGAATTATGGGTGAGACGGGCGGCTGATTCCTTACTTACGCCCAAGAGGCGGTGCTATTGGTT
TATACAACAAAATCGATGCATACCGTCTGCAAGCGAAGGATTAACACTTACGAAGCCCAACACCACTTAGGCTTTGGTGATGAC
TACCTGATTTTACGTAAGCAGCGGAATGTTACGGCTTTAGGACCCCAAAAATCCGTTTGGTGACCAACATCTTAAGAAAAT
CAATGAACCTTAAATCTTTGGTATTGAGATTGAAGAAGTGGTGAACACCTCTGCTCATATCAATCGGGTAATGAAGCTATCTTG
AA = 690 BASES

HD-9 Primer M13 forward

TCCCTTTCTTGTTCCTGTTGCCAGCAGAACGAGAACCTAAGAAGCTATCAATACAAGACTTCAACGCTTTTGCTGTAATTAAGTAA
TCTGCAAAACAGCGCATGAGCAATATCTGCATAGTGTCTACAACTTGATCTTGAGTTACTGCTGCAAAAGAAGAGCAGAGGCAAA
AATGAGTGACGAAGTACTGCTTTTGTCTACTAAAGATTTAATGGTCATGAAGCATCCTTGTGAGCTTTAAATATTATTCTGAGT
AATAGTGCAAAACCAATTATCAATTTGCACTACGGATTGGAATAATACAACTTACAAATTTTATTGCAAGATAAATACAAAAAACCTC
ACATATGTAAGGCTTTTAAACATTTTATGTTAAGCGCAACAGCAAGCAATCAGATATCTGCTTACCGTGTGAAACT
TTCGCTTCAGATAGCTTTCATTACCCGATTTGATATGAGCAGAGGTGTTACCACCTTCTCAATCTCAATACCAAAAGATTAAAG
TTCATTGATTTTCTTAGGATTTGTTGGTCACCAACGGATTTTGTGGTGCTTAAGCGCGTAACATTTCCGCTGCTTCAGTAAAA
TCACGTAAGTCATACCAAAACCTAAGTGTGTTGGCTTCGTAAGCTCTCATACCTTCCCTTTGCAGAC = 672 BASES

2. Non-haemolytic clones from transposon mutagenesis.

M2 Primer M13 forward

GCTAGAATTAGCTTTCTGACGGAATGTTAATTCGCTTGACCTGAGCACTGATGAATCCCTAATGATTTTGGTAAAAATCATT
AAGTTAAGCTGGATACACATCTTGTCATATGATCCCGGATCGGTGGAGCTCGAATTGACCGCTCCGAATTTCTGCCATTTCATCGC
TTATTATCACTTATTCAGGCGTAGGAACAGGCGTTAAGGGCACCAATRACTGCCCTTAAAAAATTACGCCCGCGCTGCCACTC
ATCGGCTACTGTTGTAATTCATTAAAGCATCTGCGGACATGGAAGCCATCACAACCGCATGATGAACCTGAATCGCCAGCGGCA
TCAGCACCTTGTGCGCTTGGTATATATTTGCCCATGGTGAAAACGGGGCGAAGAAGTGTCCATATTGGCCACGTTTAAATCA
AAACTGCTGAACCTACCCAGGGATTGGCTGAGACGAAAACATATTTCTCAATAAACCTTTAGGGAATAGGCCAGGTTTCCAC
GTAACACGCCACATCTTGGCAATATATGTTAGAACTGCCGGAATCGTCGTGTTTCACTCCAGAGCGATGAAAACGTTTCAG
TTTGCTCATGGAACCGGTGTAACAAGGGTGAACACTATCCATATACCAGCTCACCGTCTTTTCATTGCCATACGAAATCCGGATG
AGCATTATCAGCGCGGCAAGATGTGAATAAAGCGCGGATAAACTTGGCTTATTTTT

M2 Primer M13 reverse

AAGTCATTTAATAGGCAACAGTAACACATCATGTTGTTATACGTAACCTATTCACAAATATCTAAGTAACAATGAAAACACTTAA
ATTCATCAATAGTCAGAAAGAACTCGGCTCTACGCTCTCGAGAAAACGCAAAACGCCACATCACGTGTCTGATGTGGCGCTTTCAAT
ATTACTGCAAAACCTAACGCTAGGTTGGTAAAGCCTTCGACTTATAAACCCATCTCTTCAGTAGTGTTTGATTTGCTTGAGATC
CATGGCTGTGAGCTTCATCATCTGTCTGATATCACTCAGCTTAGAGTTGGCATTTATCTGTTTCTCACACGCGTCAGATTTCGCAT
CCCATGATGTGCCATCTAAGAACACATCACACGCTTTCTTAAGTGAACCAAGGTTCTGTTCAAGCTCGCTCTCTTCTTCTCAAGC
TTTTCAAGTCTTTGAGATATTTGTAATCTTTACGGAACAGCTTCACGAGAACGTTCAACAAGAGTCCGATCGCATCCGCTGACG
GTTTAGCTTTTGATTTCTTGCTCAGTTTTATCTAGCGTTTGCTTTTGTGCTTTAACTGCTGCTCTAACGAGTAGTTGCGCTTTT
CTAAACCGCGATGTTTCTGCTAATCTTTGAAGCTCTTTTGGATGCTCTTCCACTTGCTTTGCTAGCGCACTTCGACTTTGGC
GTGATTTTNGTATCGACTTTTAGCCACTTTC

M3 Primer M13 forward

GCTAGAATTAGCTTTCTGACGGAATGTTAATTCGCTTGACCTGAGCACTGATGAATCCCTAATGATTTTGGTAAAAATCATT
AAGTTAAGGTGGATACACATCTTGTCTATATGATCCCGGATCGGTGGAGCTCGAATTGACCGCTCCGAATTTCTGCCATTTCATCCG
TTATTATCACTTATTCAGCGGTAGCAACAGGCGTTAAGGGCACCAATAACTGCCCTTAAAAAATTACGCCCGCGCTGCCACTC
ATCGGCTACTGTTGTAATTCATTAAAGCATCTGCGGACATGGAAGCCATCACAACCGCATGATGAACCTGAATCGCCAGCGGCA
TCAGCACCTTGTGCGCTTGGTATATATTTGCCCATGGTGAAAACGGGGCGAAGAAGTGTCCATATTGGCCACGTTTAAATCA
AAACTGCTGAACCTACCCAGGGATTGGCTGAGACGAAAACATATTTCTCAATAAACCTTTAGGGAATAGGCCAGGTTTCCAC
GTAACACGCCACATCTTGGCAATATATGTTAGAACTGCCGGAATCGTCGTGTTTCACTCCAGAGCGATGAAAACGTTTCAG
TTTGCTCATGGAACCGGTGTAACAAGGGTGAACACTATCCATATACCAGCTCACCGTCTTTTCATTGCCATACGAAATCCGGATG
AGCATTATCAGCGCGGCAAGATGTGAATAAAGCGCGGATAAACTTGGCTTATTTTT

M3 Primer M13 reverse

AAGTCATTTAATAGGCAACAGTAACACATCATGTTGTTATACGTAACCTATTCACAAATATCTAAGTAACAATGAAAACACTTAA
ATTCATCAATAGTCAGAAAGAACTCGGCTCTACGCTCTCGAGAAAACGCAAAACGCCACATCACGTGTGATGTGGCGCTTTCAAT
ATTACTGCAAAACCTAACGCTAGGTTGGTAAAGCCTTCGACTTATAAACCCATCTCTTCAGTAGTGTTTGATTTGCTTGAGATC
CATGGCTGTGAGCTTCATCATCTGTCTGATATCACTCAGCTTAGAGTTGGCATTTATCTGTTTCTCACACGCGTCAGATTTCGCAT
CCCATGATGTGCCATCTAAGAACACATCACACGCTTCTTAAAGTGAACCAAGGTTCTGTTCAAGCTCGCTCTCTTCTTCTCAAGC
TTTTCAAGTCTTTGAGATATTTGTAATCTTTACGGAACAGTTTCACGAGAACGTTCAACAAGAGTCCGATCGCATCCGCTGACG
GTTTAGCTTTTGATTTCTTGCTCAGTTTTATCTAGCGTTTGCTTTTGTGCTTTAACTGCTGCTCTAACGAGTAGTTGCGCTTTT
CTAAACCGCGATGTTTCTGCTAATCTTTGAAGCTCTTTTGGATGCTCTTCCACTTGCTTTGCTAGCGCACTTCGACTTTGGC
GTGATTTTNGTATCGACTTTTAGCCACTTTC

M2 forward sequences

M2 Primer M13 forward (1 forward)

GGTACAAATTAGCTTTCTGACCGAATGTTAATTCCTGCTGACCCCTGAGCACTGATGAATCCCCCTAATGATTTTGTAAAAATCACTT
AAGTTAAGGTGGATACACATCTTGTCTATATGATCCCGAATCGGTGGAGCTCGAATTGACGGCTCCGAATTTCTGCCATTCATCCGC
TTATTATCACATTATTAGGCGTAGCAACAGGCGTTTAAAGGGCACCATAACTGCTTAAAAAATTACGCCCGGCTGCCACTC
ATCGCAGTACTGTTGTAATTCATTAAGCATTCCTGCCGACATGGAGGCCATCACAAACGGCATGATGAACCTGAATCGCCAGCGGCA
TCAGCACCTTGTGCGCTTGCCTATATATTGCCCCTGTTGAAACGGGGCGAGAGACTTCCTCATATTGGCCACGTTTAAATCA
AACTGGTGAACCTCACCCAGGGATGGCTGAGACGAAAAACATATTCTCAATAAACCTTTAGCGAAATAGGCCAGGTTTTCACC
GTACACGCCACATCTTGGCAATATATGTGTAGAACTGCCGGAATCGCTCGTGGTATTCACTCCAGAGCGATGAAAACGTTTCAG
TTTGCTCATGGAAACGGGTGAACAGGGGTGAACACTATCCATATACCAGCTCACCGTCTTTTCATTGCCATACGAAATTCGGGATG
AGCATTCATCAGCGCGGCAAGATGTGAATAAGGCCGGATAAAACCTTGCTTATTTT

M2 Primer internal cat (2 forward)

CTATCCATATACAGGCTCACCGTCTTTTCATTGCCATACGAAATTCGGATGAGCACTTCATCAGCGCGGCAAGAATGTGAATTAAGG
CCGATATAAATCTGTGCTTATTTTCTTTACGGCTCTTAAAAAGCCGTAATAATCCAGCTGAAAGCGCTCGGTATAGGTACATTGA
GCAACTGACTGAAATGCCTCAAAATGTTCTTTACGATGCCATTGGGATATATCAACGGTGGTATATCCAGTGATTTTCTCCAT
TTTAGCTTCTTAGCTCCTGAAATCTCGATAACTCAAAAAATAGCCCGGTAGTGATCTTATTTCAATTATGCTGAAGTTGGAAC
CTCTTAGCTGCCGATCAACGCTCATTTTTCGCCAAAGTTGGCCAGGGCTTCGCCGATCAACAGGGACACGAGATTTATTTAT
TCTGCCAAGTGATCTTCCGTCACAGGTATTTATTCGGACGCGTCTCGGTACCGGGCCCCCTCGAGGTGACGGTATCGATAAG
CTTGATATCGAATGGCGAAGCAAACTGATACATTTCGCAAGTGAGCTATCCCAATGAATCGCTTCCCATGCGAACTAATAAT
ATCTTGTTTGTTACAAATCTCGGGTGAAGTTTAACTAATACTCTAAAAAGCGAGTTTCAGGATAT

M2 Primer M2-DMC-1 forward 1 (3 forward)

TAAGTAATAACTCTAAACCGGAGTTTCACGATATCCTMTTTAATAGAACGTTCTGCTCTTATAAATACGGTTTAAATCGGGTCA
AATCTGATCATATAGCCAACGTTTATTTTATTATAATCTCTCAACAATTTAGATGACTATAAATGACTATTATATGAATTA
AGTCACATGTTTTATTTCTGGGTGTTTTTTCGGTGGTTCAAAACAAAGCATTCCTCATTTTTTATGAAGAAGCTCTCGCATCTAGT
CACAACTCGTTTAATATAAGTAATAATCTAATTAAGTTTGTGATTTTATTAGGGAGCTTTTAATAGAGAGTATGGCTTAATCTTTC
TCTCAATTTAATTAGAACGTTGCTTAATTAATCTTATTTTATTTGTTGGGTATACGATAGTAATAATTCAGTCACTTTAAGTA
TCGAATGGGTATAAAAGCCGCGCATTTGAGTGGGGCTTTGGTTCAATCAATCTCGATTATGCTTTTACAGCTTCAACATGCTTT
GAAGACGTTAAAGGTTTGAAGAACAGCTTAGTTTCTTCGATAACCAGCTGSCGTAGCAAAATAGGCCGATCAGGTTTGAATTGC
CATCAGGCGCTCACGATATCTGCCATTATCCAGATCATGTCGAGCTTTANSAATGCTCCAGAACCCACTAATGCGATGAATATTAT
CTTGTAAGGCAATACTGCTTTGGTACCTAGCAAG

M2 Primer M2-DMC-1 forward 2 (4 forward)

GAGCTTTAGAATGCTCCAGAANCCACTAATGCGATGAATATTATCTTGAAGGCAATACTGCTTTGGTACCTAGCAAGAACACAAAC
GCAGGCTCACCGTAGTAGTTCCCAACCTAAAATTTGTTGAACCGCAAGAGATCAGGCCAAGCAAAACACATAGGGCCAAAGCG
TGCTGCTGATTCAAACCAACGGCGAATGCATGAGTGGTATCGAGCAGCACCAGAGAGGTCGGTTTGGCAAGCGCCGTTAATATAAGC
GCCAAACCTGTGATGTTACAGATCATGATGTTATCGAAGAGGTACCTGTCTATAGAGACAAGGCTTGTCTCAGCGATGAATCTGT
TTTTGCGAGCGCGCGCCGCTATGGTGGCTTACCTAGACCAGATTGCTTCGAGAACACACCAGAGCGATGCTCTGATTCGATAGCAA
GCATGATGCTTTCGACCAAGAAACCCCGGTTGCTGCTGTGTGTTGAACGCGAGAGGTAAACAAAGGTTAATAGCTTCAGAGT
TGGTCTGCTTTGAATCAGAACGCTTAACATGCGACAATGTACATAACCGCCATAGTAGGAACAACCTTCCCTGCCACCTTAGC
GATAGATTGAATACCAACCAAGGTTACCACTGCGACTAGTATCGTTAGAACGACAGCAGACATCTCAC

M2 Primer M2-DMC-1 forward 3 (5 forward)

TTGCTGCTGTGTTGGTAAACGAGAGGTAAACAACAGGGTAATAGCATTCAACAGTTGCTCTGATTTGAATCAGAACGCTTAA
CATGCGACAATGTACATAACGCCCATAGTAGGAACAACCTTCCCTGCCACCTTAGCGATAGATTGAATACCAACCAAGGTTACCA
TGCCACTAGTATCGTTAGAAGCAGACAGCAGACATCTCACGAGAAGCACCAGATGAATTTGAGTTGCGTCTAAGATAGCGTTTACTT
GTGGGAAGGTACCAATACCGAAGCAGGCAACACCGAGCGGCAAGACAGCBAACATTACCGCCAGATTCTGTAACCGACACCGTAT
TGCAAGGTAGTACATTGGGCCAACCAACCTTTGCCATTGCTATTCGGTTCTCGGTTATTACAGCAAGTAGACATTCCTGCTATT
GGTGGCCATGCCAATACAGCGGCAAGCCACATCCAGATAGGGCGACCGCCCAACCTTCGATTGCGGCTGCTACCAACGA
TGTTACCTGTACCAATGGTTGCGGAAGCGGGTACACAAGCGGCAAACTTGATACGCTACCCGTTACCGGATTGTCTTGTGCTG
AATACCATTTTTAGCGCGGTTGGGAGCTGTCTAACTGAAGTAAGCCTAAGCGAAAGTAAAGTAGATACCTGTACCCACTAGCAG
AATAGCAGTGGTGGTCCCCAA

M2 reverse sequences

M2 Primer M13 reverse (1 reverse)

AAGTCATTTAATAGGCAACAGTAACACATCATGTTGTTATACGTAACCTATTCAAAATATCTAAGTAACAAATGAAACACTTAA
ATCATCAATAGTGAAGAAATCGGCTCTACGCTCTCGAGAAAACGCAAAACGCCACATCACGCTGTGTGATGTGGGCGCTTCAAT
ATTATCTAGTTTATTTAGAGCTTGGTAAAGCTTCAGCTATATAAACCCATCTTTCAGTAAGTGTTTGATTTGCTTCAGATC
CATGGTGTGGACTTCAATCATCTGCTGATATCACTCAGCTTAGAGTTGGCATTATCTTGTCTTCACACCGGCTCAGATTTCGCAT
CCCATGAGTGTCCATCTAAGAACACATCACAGGCTTCTTAAGTGAACCAAGGTTCTGTTCAAGCTCTCTCTTTCTTCTCAAG
TTTTCAAGTCTTGTAGATTTTGTATTTTACGSAACAGTTCAAGAGACCTTCAACAAAGCTCGACTGCATATCCGCTGAGC
TTTTCAGCTTTGATACCTTGTGCTTTTATCTAGCGTTTGTCTTGTCTTAACTGCTCTCAACGAGTAGTCTCCCTTTT
CTAAACCGCAGATTGTTTCTGCTAATCTTTGAAGCTCTTTTGGATGCTCTTCCACTTGTCTTGTACGCGCAACTTCGACTTTGGC
GTGATTTCTGATGACTTTTAGGCACTTTC

M2 DMC-1 reverse 1 (2 reverse)

GCTGCTCTAACGAGTATTCGCTTTTCTAAACCCGAGATTGTTTCCGTAACCTCTGAAGCTCTTTTGTAGCTCTTCCACTTGC
TTTCTAGCGCACTTCGACTTTGGCGTCGATTTCCGATTCAGACTTTAGCCACTTTCGCTTCAACGGATGTCATCAATGTGCTTTT
GCTATCAGCGAGTCTTTGATAACGAGTTTCGAGGACCTGATAAGTGGACTCCCACTTCAAGCCGTGACTGCAGAACCGATTAAAG

Primer M2-

CGGGGAAGCCCTGGGGCAACTTTTGGCGAAAATGAGACGCTTGATCGGCACGTAAGAGGTTCCAACTTTCACCATAATGAAATAAGAT
TCACTACCCGGGCGTATTTTTCGASTTATCGAGATTTTCAGGAGCTAAGCAACATAAAATGGAGAAAAAATCACTGGATATATACCAC
CGT TGAATATATCCCAATGGCATTGTAAGAACATTTTGGGCGATTTTCAGTCAGTTGCTCAATGTACCTATATACCAGACCGCTTCAGC
TGGATATTACCGGCTTTTAAAGACGTAAAGAAAAATTAAGCAAAAGTTTATCGTCGGCCCTTATTCACATTCGTGCCGCGCTGAT
GAATGCGTCATCCGGAATTTTCGATGGCAATGAAGAAGCGGTGAGGTGGATATGGGATAGTGTTTCACCCCTTGTTACACCGCTTTTCG
ATGAGGAAACTGAAACGTTTTTCATCGCTTCGAGTGAATACCCAGCAGATTTCCCGGCGAGTTTCTACACATATATTCGACAGATGTG
CGCGTGTACCGTGAAGAACTGGGCTATTTCCCTAAAGGGTTTATGGAATATGTTTTCGCTTCACGCCAATATCCCTGGGTGAGTTT

CACCAGTTTGTATTAACCGTGGCCAAATATGGACAACCTCTTCGCCCCCGTTTTCACCATGGGCAATATTATACGCAAGGCGACAA
AGGTGCTGATGCGCTGGCGATTC

M4 Primer M4 Internal 4 Forward (5 forward)

GTTTTGATTAAACGTGGCCAAATATGGACAACCTCTTCGCCCCCGTTTTCACCATGGGCAATATTATACGCAAGGCGACAAAGGTG
CTGATGCGGCTGGCGATTTCAGGTTTCATCATGCGGTTTGTGATGGCTTCCATGTGGGCAAGTGTCTTAATGAATTACAACAGTACTG
CGATGAGTGGCAGGCGGGCGGTAAATTTTAAAGGCAGTTATGGTGCCCTTAACGCGCTGGTTGCTACGCGCTGAATAAGTGATA
ATAAGCGGATGAATGGCAGAAATTCGGACGCGTCAATTGAGGCTCCACCGATCCGGGATCATATGACAAGATGTGTATCCACCTTA
ACTTAATGATTTTTACCAAAATCATTAGGGGATTCATCAGGGCTCCACTAGCGAGCAAGTGACACTTCAGGCGCGCCCAATGGTTC
CAGCCAACTCAGAGCTTCCGATTGCAATTGAGTTGTATCGTTCAAGTATCTCTACCGGTACCGATTCAACGCCGACATTAGCTAC
GATGTTGAGTTTAAACGCTTCCCTCGGTTGGGGTGGCAACGCATGGCATAACACCCAGACAACCGCCCTTACAGAGCTCACACCTT
CACTATGGGTCGCGGCAAGTGATAAATCTGCAGACATTGCTACCAATGGGATCAGGTTACATTCCAGGTGAACCAAAATGGTGGG
AATGGGGCTGGGCTATTAAAGAAGCTGGCTTATCAAGCATGCAAAAGCTTGGCACTGGGCGCTCCGTTTTTCCACGCTCCGTGGACT
GGG

M4 Reverse sequences

M4 Primer M13 reverse (1 reverse)

TTGATAAGCCAGCTTCTTAATAGCCAGCCCAATCCCAACCTTTTGTTCACCTGGAATGTAAAGGTGATCCCATTTGGTAGCGA
ATGTCTGCAGATTATPACTGCCGCGACCCATAGTGAAGGTGTGAGCTCTGTAAGGCGGTTGTCTGGGTGTGTATGCCATGCGTT
GCCACCCCAACGGAGGAGCCGTTAACTCAACATCGTACTAATGTGCGGCTTGAATCGGTACGGGTAGGAGATCTTGAACGAT
ACAACCTCAACTCGAATCGAAGCTCTCAGTTGGCTGGAACCATTTGGGCGCGCTTGAAGTGTCACTTGTCTGCTAGTGGAGCCCTGA
TGAATCCCTTAATGATTTTGGTAAAAATCATTAAAGTTAAGGTGGATACACATCTTGTCTATGATCCCGGATCGGTGGAGCTCGAA
TTGACGCGCTCGAATTTCTGCCATTCATCCGCTTATTATCATCTATTTCAGGCTACCAACGAGCGTTTAAAGGCAACCAATAACTG
CTTAAAAATAATACGCGCCCTGCCACTCAGCAGTACTTGTGTAATTCATTAAAGCATTTCCGAGCATGAAAGCCATCACA
AACGCGATGATGAACCTGAATCGCCAGCGGATCAGCACCTTGTGCGCTTGCCTATAATATTTTGGCCATGGTGNAAACGGGGGCG
AAAAAATTTGCTCATANTGGNACCGGTTTNAATCNAAACTGGNGGAACTCACCCCNGGATTTGGNTGAAACNAAAAAANAAA
NTTTTTC

M4 Primer M13 forward (2 reverse-taken from M2 sequencing)

GCTAGAATTAGCTTTCTGACGGAATGTTAATCTCGTTGACCTGAGCACTGATGAATCCCTTAATGATTTTGGTAAAAATCATT
AAGTTAAGTGGATACACATCTTGTCTATGATCCCGGATCGGTGGAGCTCGAATTTGACGCGTCCGAATTTCTGCCATTCATCCGC
TTATATATCACTTATTCAGGCGTAGCAACCGGCGTTTAAAGGCAACCAATAACTGCCCTTAAAAAAATACGCCCCCGCTGCCACTG
ATCGCAGTACTGTTGTAATTCATTAAAGCATTTCTGCCGACATGGAAGCCATCACAAACGGCATGATGAACCTGAATCGCCAGCGGCA
TCAGCACCTTGTGCGCTTGGGATATAATTTGCCCATGGTGAAGAACGGGGGCGAAGAGTTGTCCATATTGGCCACGTTTAAATCA
AACTGGTGAACCTCACCAGGGATTTGGCTGAGACGAAAAACATATTCTCAATAAACCTTTTAAAGGAAATAGCCAGGTTTTCACC
GTAAACACGCCACATCTTCCGCAATATATGTGTAGAACTGCCGGAATCGTGGTGGTATTCACTCCAGGAGATGAAACGCTTTCAG
TTTGCTCATGAAACGGTGTAAAGGGTGAACACTATCCATATACCAGCTCACCCTCTTTCATTGCCATACGAAATTCGGGATG
AGCATTTCATCAGCGGGGCAAGAATGTGAATRAAGCGCGGATAAACTTG

M4 Primer internal cat (3 reverse-taken from M2 sequencing)

CTATCCATATACAGGCTCACCCTCTTTCATTGCCATACGAAATCCCGGATGAGCATTCATCAGGCGGGCAGAAATGTGAATAAAGG
CCGAGTAAATCTGCTTATTTCTTTACCGCTTTTAAAGGGCGTAATATCCAGCTGAACGGTCTGGTTATAGGTACATTTGA
CGACTGACTGAATGCTTCAAAATGTTCTTACGATATATCAACGGTGGTATATCCAGTGATTTTTCCTCAT
TTTAGCTTCTTACGCTCCTGAAATCTCGATAACTCAAAAATACGCCCCGTAGTGATCTTATTTCATTATGGTGAAGTTGGAAC
CTCTTACGTTGCCGATCAACGCTCTCATTTTCGCCAAAGTTTGGCCACGAGGCTTCCCGGTATCAACAGGGACACAGGATTTATTTAT
TCTGCGAAGTGAATCTTCCGTCACAGGTATTTATTCGGACGCTCTCGGTACCGGCGCCCGCTTCCAGGTGCGAGGTATCGATAAG
CTTGATATCGAATGGCGAGCAAACTGATACATTTCCGCAAGTGACGTATCCCCAATGAACGCTTCCCATGCGAACTAATAAT
ATCTTGTITGTTCAATCTCGGGTGAGTTTAAAGTAATAACTCTAAAACGCGAGTTTCAGGATAT

M4 Primer Tn cat left (4 reverse)

CCGGTATCAACAGGACACAGGATTATTTATCTGCGAGTGATCTCCGTCACAGGTATTTATTCGGACGCTCTCGGTACCC
GGGCCCCCCTCGAGGTCGACGGTATCGATAAGCTTGATATCGAATTCCTGCGACCCCGGGGATCCACTAGTTCTAGAGCGGCGC
CACCCCGGTGGAGCTCCACCGATCCGGGATCATATGACAGATGTGTATCCACCTTAACTTAATGATTTTACCAAAATCATTAGG
GGATTATCAGAGTGGAGCCCCCATTTGATTTAGACAAGCTTTGGTTGGCTTGAACCTTAATGGTGGCTTATTTCCGCCAACAA
AGGCCACTTAACCAATTTCTCTGTTTGAACACTTTGTGGAAGCCATAGCTGTCTGTTTTTGAACGATTTGTGACTCATCCACT
TCAGGTCGACAACCACTTGTTCGGCGATTAATCTGTTGTTCTTGGCTGTGCGTAAACGGTCTTAACCAAGTTCAGGATCTGATTGG
ACAACATCACCATGCCAAAAGTTATCGTCTTAACGGTGTAGGCAGGTTTGTCCAGGCTGATTTTATGTTTTTCAACACAGCGATA
ACCGCTACAAGAACCACTATTTTCTTGTATACCCAACTGTCCGACAGCGCTTATTGTCTATGCTTCCCAACGCTACTGACT
GTTATTACGCCAACCCACCGGTATC

M4 Primer M4 internal 1 reverse (5 reverse)

TTATTGTCTATGCTTTCGCCAACGTAAGTGTATTATACGCCAACCCACGCTATCCAAGGTTGTGAGCTAGATAGCTTAAGTGGG
CGAACAAAATCGTTACGGTTATTTACCAACTCATATTGAACATCTAATCGCTACCTTCTGGAAGTGAATTTAGCAGAGTACTTTGG
AATCTCAGACTGGTCAATGTTTCGGATAACAGAACCTTTTCCGCTTGGTTGTATCTTGTATTATAGGCCATTTATCCCGGGCCCA
TGATACCCAGTTTCTCTTTAAGCCTGTGATCTGCCAAGTGGCCATTCAGCCAGCAAGCACTCTTTTGTCTCTGGGCTTCAAG
CGGTCTAACGGCGGATAGCCAGAGGACATACATCGTCACCAAGTTGGTCATGGAATTTTGGTCTGGGTAAATTTTCCGCTTAC
GCCAGTAGATAGAACAGACAACTGCGGTTGCTAAGAGACTTCTGTTTATTCCGATCATTAAACAATTCCTCATTTGTAAATTTT
CTATTTTGTAAATGTTTTTGTGCCATGCAAGTTAAACAAGCAAGTTTCACTTTTTCATGTTGATAAATAGATAGATACTGAAAGGG
TTGAATTAATTTACCTTTTAAATTCACCGCTTGPATTTGTTAATTTGTTTTTGGTGAAGAGGTGTAACCTAAAAGAAAATGT
TTGATGAGGCTCAACCGTTTTTAAAGAAATGAGAAGTTATGATTGATACATTAAACGTC

M4 Primer M4 internal 2 reverse (6 reverse)

GATAAATAGATAGATACTGAAAGGGTTGAATTAATTTACCTTTTAAATTCACCGGCTTGTATTGTTTAAATTTGTTTTTGGTGAA
AGAGAGTGTAACTAAAAGAAAATGTTTGTAGAGGCTCAACCGTTTTTAAAGAAATGAGAAGTTATGATGATACATTAAACGTC
AATAGTTTTTTCAGCTGGTCTATCCGAGTGAAATGAATGGTTAAGTACGAGAGGACATATTATTCACATCGTTTTTATTTGATG
TAAATATCAACAGGAAAGCGATAAATCAGATTGGATGTCTAAATTTAATCCAGTGTTTTAAACACTCGATTAAATTTGAATGCTCAA

AATACAAGAGGTGTTTATTGATGCAGTAATAAATTTAACCATCCATTTTTATGCTGTAATTCAGAACGATGTTAATTAAACAA
AGTGAATTTAACTGGTGGCCAAACGGCGACAAATTATCGTTTAGCCAAAAGTTGTGTCTTCCATTTATTACATGTCCGAATGATGG
CAACACAGATCATTGAACTTAATATATCAACCTTAAGTGTAAAGGGTCATTGTTGATAAGCAGSTAGGGCGACAACAAGCTGTCA
TTCATGGCTTTATTTATTTTTTGCACGGAGAGTAAGTCGATAGTGCCACCTATTGAAAAGAATAGGGSATGAGAAGGGCCGGCCC
TCACCTCTAAACCGTAGAACA

Appendix 4

M4 Primer M13 forward

GCCTGACCCGATGTTCTACGGTATAGAGGTGAGGCGGCGCTTCTCAATCCCTATTCTTTTCAATAGCTGGCACTATCGACTTACTC
TCCGTGCAAAAAATAATAAAGCGATGAATGACAGCTTGTGTGCGCCCTACCTGCTTATCAACAATGACCCTTAACACTTAAGGT
TGATATATTAAGTTCBAATGATCTGTTTSCCATCATTCGGACATGTAATAATGGAAGAACACAACCTTTGGCTAAACGATAATTTG
TCGCGCTTTGGCCACCAGTTAAATTCACCTTGTGTTTAAATTAACATCGTTCCTGAATATTACACATAAAAAATGGATGGTTAAATTTA
TTACTGATCAATAAACACCTCTTGTATTTTGAGCATTCATTAATCGAGTCTTTAAACACTGGATTAATTTAGACATCCAAAT
CTGATTATCGCTTTCTGTTGATATTTACATCAAAATAAAACGATGTGATAATATGCTCCTCTCGTACTTAACCATTCATTTT
ACTGCGATAGCCACGTCAAAACATTTTACGCTTAATGATCAATCACTCTCTATTTTCTAATAACCGTTTGAGCCCAT
CAAAACATTTTCTTTTATGTTACACTCTCTTTTACCATAAAACAAATTAACAATACAGCCGTTGAATTTAAAGGTAAATTAAT
CAACCCCTTTTCAGTATCTATCTATTTTATCAACATGAAAAAGTG

M4 Primer M4 internal 1 forward

CCAAAAACCAATTAACAATCAACCCGTTGAATTTAAAGGTAAATTAATPCAACCCTTTTCACTATCTATCTATTTATCAACA
GAAAAAGTGAACCTTGCTTTGCTTTAACTGCAATGGCACAATAAACATATATCAAAATAGAAAAATTACAAATGAGGGAAATTTGTAAATGA
TCCGAATAAACAGAGTCTCTTACCAACCGCAGTGTGTCTGTTCTATCTACTGCGGTAAACGCGAAAAATTTACCCAGACCAATA
GTCCATGACCAACTTGTGTAGCATGATGTCTGCTCTGGCTATCGCCCGTTAGACCGCTTTGAAGCCGAGAGCAAAAGAGTGTGTTT
GCTGGCTAGAAATGGGCACTTGGCAGATCACAGGCTTAAAGAGAACTGGGTGATCATGGCCCGGGATATAATGGCTTATTAAAC
AAGATACACCAACGCGCAAAACGTTCTGTATTCCGAACAATGACCGAGTCTGAGATTCCAACTACTCTGTAAATCAGTTCAGAA
GGTAGCGATTTAGATGTTCAATATGAGTTGGTAAATAACCGTAACGATTTTGTTCGCCCACTAAGCTATCTAGCTCACAAACCTTGG
ATACCGGTTGGGTTCGCGGTAAATAACACTCAGTACGTTGGCGAAGACATGACAAATAGGCGCTCTGGCGACAGTTGGGTGATACAG
GAAACAATGGTGGTTCTGTAGCGGTTATCGCTGTGGTGAATAAATAAATCAACCGTCGACAACCTTTGCCATACCCGTTAAACGAC
GATAACTTTTGGCATGG

M4 Primer M4 internal 2 forward

GCCTACACCGTTAACGACGATACCTTTTGGCATGGTGTATGTTGTGGAATCAGATCGTGAACCTGGTTAAGACCGTTTACGCAACAGC
AAGAAACACAGTAAATATCGGCCAACAAGTGGTGTGCGACCTGAAAGTGTGATGAGTCAACAACTGGGTCAAAAACGAACAGCTATG
GGTTCTCACAAAGTGTTCAAAACAGAGAAATACGTTTAAAGTGGCTCTTGTGCGGAACTAAGCTACCAATTAAGTTGGAAGCCCAAC
CAAGCTTCTCTAATCAAAATGGGGGCTCCACTCTGATGAATCCCTAATGATTTTGGTAAAAATCATTAAAGTTAAGGTGGATACA
CATCTTGTCTATATGATCCCGGATCGGTGGAGCTCCACCCCGGTGGCGCGGCTCTAGAACTAGTGGATCCCCCGGGCTGAGGAAT
TCGATATCAAGCTTATCGATACCGTTCGACCTCGAGGGGGGGCGGCTACCGAGGAGCGCTCCGATATAATACCTGTGACGGAAGAT
CACTTTCGCAATAAATAAATCCTGGTGTCCCTGTTGATACCGGAAGCCCTGGGCCAATTTTGGCGAAATGAGACCTTGATCG
GCAGTAAGAGGTTCCAACTTTCCACATAATGAATAAGATCACTACCGGCGGTATTTTTCAGCTT

M4 Primer M4 internal 3 forward

CCGGGAAGCCCTGCGCCCAACTTTTGGCGAAATGAGACGTTGATCGGCACGTAAGAGGTTCCAACTTTTACCATAATGAATAAGA
TCCTACCGCGGCTATTTTGTAGCTTATCGAGATTTTCAGGAGCTAAGGAAGCTAAATGGAGAAAAAATCACTGGATATACCAC
CGTTGATATATCCCAATGGCATCGTAAGAATCAATTTTGAAGCATTTCAAGTCAGTTGCTCAATGTACCTATAACAGACCGTTTCCAGC
TGATATTTACGGCTTTTAAAGACCGTAAAGAAAAATAGACCAAGTTTTATCCGGCTTTATTCACATCTTTTGGCCCGCTGTATG
AATGCTCTCCGGAATTTCTGTATGGCAATGAAAGACGGTGGAGCTGGTGTATGGGATAGTCTTACCCCTTGTTCACCCCTTTTCCA
TGACCAACTGAAACGTTTCTATCGCTCTGGAGTCAATACCACGACGATTTCCGGCAGTTTCTACACATATATTGCAAGATGTGG
CGTGTTCAGGTTGAAACCTGGGCTATTTCCCTAAAGGGTTTATGAGAAATATGTTTTCGCTCTAGCCCAATCCCTGGGTGAGCTTC
ACCACTTTTGAATTAACGTTGGCCAAATATGGACAACCTTCTTCCGCCCGGTTTTCACCATGGGCAATATTATACGCAAGGCGACAA
GGTGCTGATGCCGCTGGCGATTCA

M4 Primer M4 Internal 4 Forward

GTTTGTGATTTAAACGTTGGCCAAATATGGACAACCTTCTTCCGCCCGGTTTTCACCATGGGCAATATTATACGCAAGGCGACAAGGTG
CTGATGCCGCTGGCGATTACGTTTCACTATGCCGTTTGTGTATGGCTTCCATGTCCGCAGAAATGCTTAATCAATTACAACAGTACTG
CGATGAGTGGCAGGCGGGGGCGTAAATTTTAAAGGCAATTTGATGGCTTAAACGCGCTGGTTGCTACGCGCTGAATTAAGTAT
ATAAGCGATGAAATGGCAGAAATTCGAGCGGCTCAATTCGAGCTCCACCGATCCGGGATCAATGACAGATGATGATACCGCTTA
ACTTAATGATTTTACCAAAATCATTAGGGGATTCATCAGGGCTCCACTAGCGAGCAAGTGACACTTCAAGCGCGCCCAATGGTTC
CAGCAACTCAGAGCTTCCGATTCGAGTTGAGTTGATCGTTCAAGTATCTCTACCCGTACCGATTCAACGCCGACATTTAGCTAC
GATGTTGAGTTTAAACGGCTTCTCCGTTGGGGTGGCAACGCTATGGCATACACACCCAGACAACCGCCCTTACAGAGCTCACACCTT
CAGTATGGGTCCGGGCGAGTGAATAATTCGAGACATTCGCTACCAATGGGATCACCGTTTACATTCCAGGTGAACCAAAATGGTGGG
ATTGGGGCTGGGCTATTAAAGAGCTGGCTTATCAAGCATGCAA

M4 Primer M13 reverse

TTGATAAGCCAGCTTCTTTAATAGCCGACGCCCAATCCACCATTTTGTTCACCTGGAAATGAACGGTGTATCCCATTTGGTAGCGA
ATGTCCTGCAGATTTATCACTGCGCGGACCCATAGTGAAGGTGTGAGCTCTGTAAGGCGGTTGTCTGGGTGTGTATGCCATGCGTT
GCCACCCCAACGAGGAGGCGGTAAACTCAACATCGTAGCTAATATCGCGCTTGAATCGGTACGGGTAGGAGATACCTGAACGAT
ACAACTCAACTCAATCGGAAGCTCTGAGTTGGCTGGAACTATGGCGCGCTTGAAGTGTCACTTGTCTGCTAGTGGAGCCCTGA
TGAATCCCTAATGATTTTGGTAAAAATCATTAAGTTAAGGTGGATACACATCTTGTCTATGTATCCCGGATCGGTGGAGCTCGAA
TTGACCCCTCCGAATTTCTGCCATTCACTCCGCTTATATCACTTATTCAGGCGTAGCAACCGAGGCTTTAAGGGCACCATAACTG
CCTTAAAAAATTAACGCCCGCCCTGCCACTCATCCGAGTACTGTTGTAAATTCATTAAAGCATTCGCGGACATGGAAGCCATCACA
AACGGCATGATGAACCTGAATCGCCAGCGGCATCAGCACCTTGTGCGCTTGGCTATAATATTTTGGCCATGTTGAACACGGGGCG
A

M4 (M2) Primer M13 forward

CTGATGAATCCCTAATGATTTTGGTAAAAATCATTAAAGTTAAGGTGGATACACATCTTGTCTATGATCCCGGATCGGTGGAGCT
CGAATTGACGCGTCCGAATTTTGGCCATTATCCGCTTATATCACTTATTCAGGCGTAGCAACCGAGGCTTTAAGGGCACCATA
ACTGCGCTTAAAAAATTAACGCCCGCCCTGCCACTCATCGAGTACTGTTGTAATTCATTAAAGCATTCGCGGACATGGAAGCCAT
CACAAACCGCATGATGAACCTGAATCGCCAGCGGCATCAGACCTTGTGCGCTTCCCTATTAATTTGGCCATGGTGAACACCGGG
GCGAAGAGTTGTCCATATTGGCCACCTTTAAATCAAAACTGGTGAACCTCACCGAGGATTCGCTGACAGCAAAACATATTTCTC
AATTAACCCCTTTAGGGAATAGCCGAGCTTTTACCCTTAACAGCCACATCTTGGCAATATATGTTGATGAACCTGCCGGAATCTG
CGTGGTATTCATCCAGAGCGATGAAACGTTTCAGTTTGTCTATCGAAACCGGTGTAACAAGGTGAACACTATCCCATATACCC

AGCTCAGCGTCTTTTCATTGCCATACGAATTNCGATGAGCATTTCATCANGCGGGCAAAGAATGTGAATAAANGCCGGATAAAAC
TTG

M4 (M2) Primer internal cat

CCATATCACCAGCTCACCGCTCTTTTCATTGCCATACGAATAATCCGGATGAGCATTTCATCAGCGGGCAAAGAATGTGAATAAAGGCGG
GATAAACTTTGTGCTTATTTTCTTTACGGTCTTTAAAAAGGCGGTAATATCCAGCTGAACGGTCTGGTTATAGGTACATTGAGCA
ACTGACTGAAATGGCTCAAAATGTTCTTTACGATGCCATTGGGATATATCAACGGTGGTATATCCAGTGATTTTTTCTCCATT
AGCTTCTTAGCTCCTGAAATCTCGATAACTCAAAAAATAGCCCGGCTAGTGATCTTATTTCAITATGGTGAAGTTGGACCTC
TTACGTGCCGATCAACGTCTCATTTTCGCCAAAAGTTGGCCAGGGCTTCCCGGTATCAACAGGGACACCAGGATTTATTTATCT
GCGAAGTGATCTTCGTCACAGGTATTTATTCGGAGCGCTCTCGTACCGGGGCCCCCTCGAGGTGACGGTATCGATAAGCTT
GATATCGAAT

M4 Primer Tn cat left

CCGGTATCAACAGGGACACAGGATTTATTTATTCGCGAAGTGATCTTCCGTACAGGTATTTATTCGGACCGCTCTCGGTACC
GGGCCCCCTCGAGGTGCGAGGTATCGATAAGCTTGATATCGAATTCCTGACGCCCGGGGATCCACTAGTTCTAGAGCGGCCGC
CACCCGGTGGAGCTCCACCGGTCCGGGATCATATGACAAGATGTGTATCCACCTTAATCTAATGATTTTTACCAAAATCATTAGG
GGATTCATCAGAGTGGAGCCCCATTGATTTAGAGAAGCTTTGGTGGCTTCGAACCTTAATGGTGAGCTTAGTTTCGCCAACAG
AGGCCACTTAACGTAATCTCTGTTTGAACACTTTGTGAGAACCCATAGCTGTTCCTTTTGACAGGTTTGTGACTCACTCCACTT
TCAGGTGACACCCACTTGTTCGGCGATATTACTGTGTTTCTTGGCTGTTCGGTAAACGGTCTTAACAGGTTCAGGATCTTATTCG
ACAACATCACCATGCCAAAAGTTATCGTCTTAACGGTGTAGGCAAGTTGTGACGGGTGATTTTAGTTTTTTCACACAGCGATA
ACCGCTACAGAACACCATTTGTTTCTTGTATCACCAACTGTTCGCCAGAGCGCTTTATTTGTCTGTCTTCCCAACGTAAGT
GTTATTACGCCAACCCACGGGTATC

M4 Primer M4 internal 1 reverse

TTATTGTCTATGCTTTCGCCAAGCTACTGACTGTTATTTACGCCCAACCCACGCTATCCAAAGTGTGAGCTAGATAGCTTAGTGGG
CGAACAAAATCGTTACGGTTATTTACCAACTCATATTGCAACTCTAAGCTCGCTACCTTCTGCACTGATTTAGCAGAGTAGTTTGG
AATCTTCAGACTCGCTCATTTGTTCCGATAACAGAACGTTTTCGGCTTGGTTGATCTTGTTTAATAAGGCCATTAATCTCCGGGCCA
TGATCACCCAGTTTCTTTTAAGCCTGTGATCTGCCAAGTGCCCATTTCTAGCCAGCAAGCACTCTTTTGGCTCTCGGGCTTCAAG
CGGTCTAACGGGCGATAGCCAGAGCGACATACATCGTCACCAAGTTGGTCTAGGACTATTTGGTCTGGGTAAATTTTCGGCTTTC
CCAGTAGATAGAACAGACAACTCGGTTCTAAGAGACTCTCTGTTTATTCGGATCATTAACAATTCCTCATTTTGTATTTT
CTATTTTGATAATGTTTTTGTGCCATGCAAGTTAACAGACAGTTTCACTTTTTCATGTTGATAAATAGATAGATACTGAAAGGG
TTGAATTAATTTACCTTTTAAATTCACGGCTTGTATTTGTTTAATTTGTTTTCGGTGAAGAGAGGTGTAATAAAGAAATGT
TTGATCAGGCTCAAACCGTTTTTAGAAAATGAGAATTTATGATGATACATTAACGTC

M4 Primer M4 internal 2 reverse

GATAAATAGATAGATCTGAAAAGGGTTGAATTAATTTACCTTTTAAATTCACCGCTTGTATTGTTTAAATTTGTTTTTCGTGAA
AGAGAGTGTAACTAAGAAAGAAATGTTTGTATGAGGCTCAACCGTTTTTAGAAAATGAGAAGTTATGATTTGATACATTAACGTC
AATAGTTTTCAGCTGGTCTATTCGGAGTGAAATGATGTTAAGTACGAGAGAGACATATTATTCACATCGTTTTTATTTGATG
TAAATATCAACAGGAAGCGGATAAATCAGATTGGATGTCCTAATTTAATCCAGTTTAAACACTCGATTAATTTGAATGCTCAA
AATACAGAGAGTTGTTTATGATGCAGTAATAAATTTAACCATCCATTTTTATGTGTAATATTAGAAGCATGTTAATTAACAA
AGTGAATTTAAGTTGGTGGCCAAACGCGACAAATATCGTTTACGCCAAAAGTTGTTCTTCCATTTATACATGTCGGAATGATGG
CAACAGATCATTTGAAGTTAATATATCAACCTTAAGTGTAAAGGGTCATTGTTGATAAGCAGGTAGGCGCACAAACAGCTGTCA
TTGATCGCTTTATTTATTTTTCGACGAGAGTAAGTCGATAGTCCAGCTATTGAAAAGAAATAGGGATTGAGAAGGGCCGGCTC
ACCTCTAAACCTAGAAC

M4-TOPO-A Primer M13 reverse

TCCCATCAACGCTCATTTTCGCCAAAAGTTGGCCAGGGCTTCCCGGTATCAACAGGGACACCAGGATTTATTTATCTGCGAA
GTATCTTCCGTCACAGATTTTATTCGGACCGCTCTCGGTACCGGGCCCCCTCGAGGTGCGACGGTATCGATAAGCTTGATAT
CGAATTCAGCTTCATCTAGCGAGTGTGTATACGCAGCTCTGAAGGACCGCCGACAGAACGGGTGAACACTTTCATCTAGTCGG
CCGTGTTCAAAATCGAATTTGTAAGAGCTTCATCACTTCTAGACCAAGTGCTTTTGTCTCTGTCGAGTATAGATACAGATGGCG
GTTAAATGCTCGCTGACTGTTTTTCGATGACTTCGTCAATCAGGTTTGGTAGCCAGGTTTAAAGCTCTGCAACAAAGATATCAA
GCCAGCAGAGCTGCTGCCCGGTTCTGATATCCCGGTTACAGCATCTGATCAAGGGGTTAAGTTAGCGGCTCTGCCGGATTTGTTCT
TCTTCGGCGGATAGCTCAACCACTTCACGCCAGTCTTTTCAAACCAACCCAGTCGTTTTCCCGACGTTGGCTACGCCACGCATG
TTCATATTTTGAACCGGTAGAGATTTTCGTTCAACCAAGTTTTCGTGAA

M4-TOPO-A Primer M13 forward

TTTTCGTCTCAGCCAAATCCCTGGGTGAGTTTCACCACTTTTGATTTAAAGCTGGCCAATATGGACAACCTTCTTCGCCCGCTTTT
CACCATGGGCAATATTTATACGCAAGGCGACAAAGCTCTGATCCCGCTGGCGATTTCAGGTTTCATCATSCCGTTTGTGATGGCTTCC
ATCTCGGCAGAAATGCTTAATGATTAACAACAGTACTGCGATGAGTGGCCGGGCGGGGCTAATTTTTTAAAGGCACTTATTTGGTGC
CCTTAAACGCTGTTTGTACGCTGATATAGTGAATAAAGCGGATGAATGGCAGAAATTCGGACCGCTCAATTCGAGCTCCACC
GATCCGGGATCATATGACAAGATGTGTATCCACCTTAATTAATGATTTTTTACCAAAATCATTAGGGGATTCATGAGGGCTCCACT
AGCGAGCAAGTGACACTTCAAGCGCGCCCAATGCTTCCAGCCAACTCAGAGCTTCGGATTCGAGTTGATGTTGATCGTTCAACTAT
CTCCTACCCGTACCGATTCAACGCCGACATTAGCTACGATTTGAGTTTAAAGGCTTCCCGCTTGGGGTGGCGAGCATATGGCAT
CACACCCAGACACCGCCCTTACAGAGCTCACACCTTCACTATGGTTCGGCGAGTGATAAATCTGCAGACATTCGCTACCAATGG
GGATCACGTTACATTCAGG

M4 TOPO A Primer M4 TOPO A1 forward

AACCAAAATGGTGGGATTTGGGGCTGGGCTATTAAAGAGCTGCCTTATCAAGCATGCAATACGCGACTGGCGGAAGCTTACGTCCA
TTCACCTCTTATCTATCGGGTGACTTCTACGCGAGCTCTCAATTTCCAGCTACTATTGAGATTTGGCCAAGCGACACCGATTATCAA
CAATCTACGTAGCAAAAGAGTTACAGATTCCGTCACGAAAGCACTGAACGATTTGGCGATATTGAGTACGCAACCAACTTCATG
CTGATGAGTTTCACTGACTTACGTTTTCGAAGGTGCTGAGATGAACATCAGCGTCTGTTGAATAACCAATGACTCACCTTTAACACTT
AATTTGCCAAAACACTCGATTACCGTAAAGACAAAGCCACGACAGCTCGTGGGCTTTCATATATTCATGTTCAAGTAGAAAA
CAGTGAAGTACACAGGCTCTTCCATTAGAAGCTTAACGCTTACGCCCACTAGTACCATSCCAGTCAACGCTTCCATCCCACTTCATA
AACTCCGATTTCTGAGCAAGTTTTCAGCGAGTTCAATGCAACGCTAAACCACTTTCACACCATTCGAATCAATCAAGTGGAC
TGAAGCCATCACATAGATTGCAATAAAGGCGAGCTTTCAGGGTTTACAAATTCAGSCAGAAAAGCCAAATAGAAAACCGCGCTT
TTGGGTTAAGCACTTAGATAAGAAACCTTACGCAAGAGACGTTTTCACATGTAGGCTTGATGAGTTTGTCTGCCAACCTTCAG
CCGCGCCATTTTCATCAAGCTCGTAACTGCTTAA

M4 TOPO A Primer M4 TOPO A1 reverse

GCTTCTTCGGCGATAGCTCAACCACTTCACGCCAGTTCTCTTTCAAAACCAACCCAGTCGTTTCCCCACGTTGGCTACGCCACGCA
 TGTTCACATTTCGAACCCGCTAGAGATTTCGCTTCAACCAGTTTCTCTGGAAGTAGGTTAGCTTGTGCCATTGGGCTTTGATTTT
 ACGTAGGGACGATTGTTGCTCGTTATTTCAGTGTTCGTTTTCAGGCTCTGCAATCCCAATCGCCGAGTTGTGGCTGAGTCATTAGAC
 CGTGAATATGAACAGAAAGCTCGGCCATCGCTTCACTACGTCCTTGATTGCCGCCAGCGGCGATCATGGAAGCTTCCTCCCAACCA
 CAAATAGAGGCTAGGTGCTGAAAGCGTGAACATTTTIGAGAGTGCTCGACTAGTTTITGAATGGGCTCATTGACTACTCTTAAT
 TEGGTCTCTTTAAATGACCCATTAAGGCTGCATCAGTTTAAGGCGATCTCGATGTTTAGTACTTTGAAGTTGGTGCATTTCAGTTT
 TTTCTGAGGCTGTTCTCTCTCAGTAGAAGATATAGCTTAACAAGGTAGGGCAGTGATATGCCAGTAATTTACTATTGTATTACAAA
 TTAACATGTACATTGTCGACAAATCAGTACTTGTATAGCTGGTGGCAATTGTTGCTTGGCAATTGTCAGATACACGGTATTG
 ATGATAGATCGCTGAACAAGAAATGCAATGGATATGTTGAATTTGAGGCATTTCATATTGCCATCATTATTTTAACG
 CTAACGCCGGGTTTATAGTACGGCATTTGGTAATTCGTAATACCAAGTCGATCTGGCTTATCTGACGGTGAATGACCA

M4TOPO A Primer M4 TOPO A2 forward

CATCGCAATCATATAAGTGGACTGAAGCCATCAACATAGATTGCAATTAAGGCGAGCCTTCAGGGTTTACAAATTAAGGCAGAAAAG
 CCAATAGAAAACCGCGCTTTTGGGTAAACACATAGATAAGAAACCGTCACGCAAGAACGCTTTGCACTGTAGCTTGTATGA
 CTTCGCTCGCCACCTTCAGACCGCGCCATTTTCATCAAGCTCGTAACTGCTTAAGCCAGCCAAATTAAGTAACCGGACC
 CACCATTTTAACTGCTGAAGAGATTTCAGCGGACTGAGCAAGAAATGCGAGAGATACCCACCGCAGAGAAAAAGCGTCACATAAA
 GACCGCTACAGATACCAAGCTGGTCATTACACCGTCAGATAAGCCAGATCGACTGGTATTAGCAATFACCAATGCGGTATCTAAA
 CCGCGGCTTAGCGTTAAATAGTGTAGGCAATTAGAAATGCCTCAAAATTAATCCATATCATTTGTCATTCTCTTCAGCGA
 TCTATCATCAATACCGTGTATCTGAACAAATTCGCAAGCAACAAATTCGCCACCGCTATACAACTCAGTATTGTTGTCACAAATGGTA
 CAGTTTAAATTTGTTAACAATAGTAAATTAAGTGCATATCACTGCCCTACCTTGTTTAAGCTATATCTCTACTGAGAAGAACAGC
 CTCAGAAAACAAAGTGGAAAGTGCACCACTTCAAAAGTACTAATCATCGGAGATGCCCTTAAACTGGATGACAGC

M4TOPO A Primer M4 TOPO A2 reverse

TTTAGTACTTTGAAGTGGTCACTTCACCTTTGTTTCTGAGGCTGTTCTTCTCAGTAGAAGATATAGCTTAACAAGGTAGGGCAGT
 GATATGCCAATAATTTACTATTGTTAACAAATTAAGTGTACCATTTGTCACAAATCAGTACTGTTATAGCTGGTGGCAATTTG
 TTGCTTGGCAATTTGTTAGATACACGGTATGATGATAGATCGCTGAACAAGATGACAAATGATGATGATATTTGAACTTTGAGG
 CATTTCTAATTTGCCATCACTATTTTAAAGCTTAACGCCGGGTTTAGATACGGCATTGGTAATTCGTAATACCAAGCTCGATCTGGCTTA
 TCTGACGGTGTAAATGACCAAGCTTTGGTATCTGTAGCGCTTTTATGTCAGCGCTTTTCTCTGCGGTGGGTATCTTCGCGATT
 TGGCTCAGTCCGCTGAACCTTTTCAAGCAGTTAAATGGTGGGTGCCGTTTACCTAATTTGGCTTTGGCTTAAGCAGTTTACGAGCTT
 TCAATGAAAATAGCGCGGCTGTGAAGGTTGCGGAGCAAACTCATCAAGCCTACAGTGCAAAACGTTCTTTGCGTGGAGGTTTCTTA
 TCTAATCTGCTTAACCCAAAACCGCGGTTTCTATTTGGCTTTTCTGCTCAATTTGTAACCCCTGAAGGCTCGCTTTATTCGA
 ATCTATCTGATGGGTTTCAGTCCATTTTATGATGCTGTGGCAATGTGGTTTAGCTGGTGCATTGAACTCGGCTAAAAA

M2/M4 A1 Primer M13 forward

CATCGCAATGATGGCTTCTGGAGCATTCCTAAAGCTCGACATGATCTGGATAATGGCAGATATCGTGAACGGCTGATGGCAATTC
 CAAACCTGATCGGCTTATTTTGTACGCCACGCTGGTTATCGAAGAACTAAGCTGTTCTTCAAAACCTTTAAGCTCTTCAAAACGAT
 TGTGAAGCTGTTAAAGCATAATCAGAAATGATTGAACCAAGCCGCGACTCAAATGCGGGCTTTTAAACCATTCGATAGCTTAA
 ACTGACTGAATTAATCTATCTGATACCCACAAATAAAATAAGATTTTAAATTAGAGCACTGTTCTAATTAATTAAGAGCAAAAGAT
 TAAGCCAAATACCTCTCTAATTAAGAGCTCCCTAATAAAATCAAACTTAATTAAGATATTACTTATATTTAACAGATTGTGACTAGAT
 CGCAGAGTTTCTTTCAATAAAATTAAGAAATGCTTTTGTGTAACCAAGCAAAACACACCAGAAATAAACATGTGACTTTAATTC
 ATATAATAGTCAAGTTTATAGTCATCTAATTTGTTGAGAGAAATTAATAAAATAAAACGTTGGCTATATGATCAGATTGACCCG
 ATTAATAAACCGTATTTATAACGACGACGCTTCTATTAATAA

M2/M4 A1 Primer M13 reverse

ATGTCGCAATGATGGCAACAGATCAATTTGAACCTTAATATATCAACCTTAAGTGTAAAGGGTCATTGTTGATAAGCAGGTAGGGC
 GACAACAAGCTGTCAATTCATCGCTTATTTTATTTTTCGACGGAGAGTAAGTCGATAGTCCAGCTATTGAAAAGAAATAGGGATTG
 AGAAGGGCCGGCTCACCTCTAAACCGTAGAACAATCGGCTCGGGCATGCTGTCATCTTGGACGGACATTAGGTGTAGAACCCTCGT
 TATCGCTTATTTTGGTGAAGTCCATCGGTTCTGAATACATTTCCATCTAACAACAAGATCTACCAGCTATGTTGATAACGGTGGCTCT
 TCTTGGGGTGGAGAAGGAATACTCCATATACCAATCAATAACGCTAACGAGGAAACAGAGCTACAAATGGCATCATAATTTGG
 CCTCTCTTTAGGAATAATTTCTATCTCTTCACTCGCTCGGCTTAATTTGCGTGGCCGTGTAAAAGATATTGATGGACTACGCTGCTGT
 CTGTAGAGTATGGAGAGTTTGAATAGCGGGCTCATCGCTGATGTATGCCACGCAATCATAATGGTATTTGTAGAGAAATAGCCC
 TCTAGTCTGGGCTTAAGGACACCTT

M2/M4 B1 Primer M13 forward

CATCGCAATGATGGCTTCTGGAGCATTCCTAAAGCTCGACATGATCTGGATAATGGCAGATATCGTGAACGGCTGATGGCAATTC
 CAAACCTGATCGGCTTATTTTGTACGCCACGCTGGTTATCGAAGAACTAAGCTGTTCTTCAAAACCTTTAAGCTCTTCAAAACGAT
 TGTGAAGCTGTTAAAGCATAATCAGAAATGATTGAACCAAGCCGCGACTCAAATGCGGGCTTTTAAACCATTCGATAGCTTAA
 ACTGACTGAATTAATCTATCTGATACCCACAAATAAAATAAGATTTTAAATTAGAGCACTGTTCTAATTAATTAAGAGCAAAAGAT
 TAAGCCAAATACCTCTCTAATTAAGAGCTCCCTAATAAAATCAAACTTAATTAAGATATTACTTATATTTAACAGATTGTGACTAGAT
 CGCAGAGTTTCTTTCAATAAAATTAAGAAATGCTTTTGTGTAACCAAGCAAAACACACCAGAAATAAACATGTGACTTTAATTC
 ATATAATAGTCAAGTTTATAGTCATCTAATTTGTTGAGAGAAATTAATAAAATAAAACGTTGGCTATATGATCAGATTGACCCG
 ATTAATAAACCGTATTTATAACGACGACGCTTCTATTAATAA

M2/M4 B1 Primer M13 reverse

ATGTCGCAATGATGGCAACAGATCAATTTGAACCTTAATATATCAACCTTAAGTGTAAAGGGTCATTGTTGATAAGCAGGTAGGGC
 GACAACAAGCTGTCAATTCATCGCTTATTTTATTTTTCGACGGAGAGTAAGTCGATAGTCCAGCTATTGAAAAGAAATAGGGATTG
 AGAAGGGCCGGCTCACCTCTAAACCGTAGAACAATCGGCTCGGGCATGCTGTCATCTTGGACGGACATTAGGTGTAGAACCCTCGT
 TATCGCTTATTTTGGTGAAGTCCATCGGTTCTGAATACATTTCCATCTAACAACAAGATCTACCAGCTATGTTGATAACGGTGGCTCT
 TCTTGGGGTGGAGAAGGAATACTCCATATACCAATCAATAACGCTAACGAGGAAACAGAGCTACAAATGGCATCATAATTTGG
 CCTCTCTTTAGGAATAATTTCTATCTCTTCACTCGCTCGGCTTAATTTGCGTGGCCGTGTAAAAGATATTGATGGACTACGCTGCTGT
 CTGTAGAGTATGGAGAGTTTGAATAGCGGGCTCATCGCTGATGTATGCCACGCAATCATAATGGTATTTGTAGAGAAATAGCCC
 CTAGTCTGGGCTTAAGGACACCTT

M2/M4 A1.5 Primer M13 forward

CAACACTCGGGTGTGAAGAGACTCTGTTTATTCGGATCATTAACAATTCCTCATTTGTAATTTTCTATTTTGTAAATGTTT
 TTTGTCATGCGAGTTTAAACAAGACAAGTTCATTTTTCATGTTGATTAATAGATAGATCTGAAAAGGGTTGAATTAATTTACCTTT
 TAAATTCACCGGCTTGATTTGTTAATTTGTTTTCGTTGAAGAGAGTGAATTAATAAGAAATGTTTGTATCAGGCTCAACCG

TTTTAGAAAAATGAGAAGTTATGATTGATACATTAAACGTCABAATAGTTTTCACGCTGGTCTATCCGAGCTGAAATGAATGGTTAA
GTACGAGAGGAGACATATTATTCACATCGTTTTTATTTGATGTAAATATCAACAGGAAAGCGATAAATCAGATTGGATGTCTAAA
TTAATCCAGTGTTTTAAACACTCGATTAAATGAATGCTCAAAATACAAGAGGTGTTTATTGATGCAGTAATAAATTAACCATCC
ATTTTTATGTGTATATTTCAGAACGATGTTAATTAACCAAGTGAAATTAACCTGGTCCCAAAACGCGACAAATATCGTTTA
GCCAAAGTGTGTCTTCCATTATTACATGTCGGAATGA

M2/M4 A1.5 Primer M13 reverse

GCCACGTGGTTATCGAAGAACTAAGCTGTTCTTCAAACCTTTAACGTCCTTCAAACGATTGTGAAGCTGTAAAAGCATAATCAGAA
TTGATTGAACCAAGCCCGCACTCAAAATGCGGCTTTTATATACCATTGATACCTTAACCTGACTGAATTTATTACTATCGTATAC
CCACAAATAAAATAGATTTTAATTAGAGCACTGTTCTAATTAATTAGAGACAAGATTAAGCCAATACTCTCTAATTAAGCT
CCCTAATAAATCAAACTTAATTAGATATTACTTATATTTAACAGATTGTGACTAGATCGCA

M2/M4 B1.5 Primer M13 forward

CAACACTGCGGTTGCTAAGAGACTTCTGTTTATTCCGNTCATTAAACATTCCTCATTTTGTAATTTTCTATTTTGATAATGTTT
TTGTGCCATGCAGTTAACAAGACAAGTTCACCTTTTTCATGTTGATTAATAGATAGATACGTAAGGGTTGAATTAATTTACCTTT
TAAATTCAACGGCTTGTATTCTTTAATTGCTTTTGGTGAAAGAGAGTSTAACATAAAAGGAAATGTTTGTATGAGGCTCAAAACCG
TTTTAGAAAAATGAGAAGTTATGATTGATACATTAAACGTCABAATAGTTTTCACGCTGGTCTATCCGAGTGAAATGAATGGTTAA
GTACGAGAGGAGACATATTATTCACATCGTTTTTATTTGATGTAATATCAACAGGAAAGCGATTAATCAGATTGGATGTCTAAA
TTAATCCAGTGTTTTAAACACTCGATTAAATGAATGCTCAAAATACAAGAGGTGTTTATTGATGAGTAATAAATTAACCATCC
ATTTTTATGTGTATATTTCAGAACGATGTTAATTAACCAAGTGAAATTAACCTGGTGGCCAAACGCGACAAATTAACCTTAG
CCAAAGTGTGTCTTCCATTATTACATGTCGGAATGGATGGCAACAGATCAATTTGAACCTTAATATATCAACCTTAAGTGTTTA
A

M2/M4 B1.5 Primer M13 reverse

GCCACGTGGTTATCGAAGAACTAAGCTGTTCTTCAAACCTTTAACGTCCTTCAAACGATTGTGAAGCTGTAAAAGCATAATCAGAA
TTGATTGAACCAAGCCCGCACTCAAAATGCGGCTTTTATATACCATTGATACCTTAACCTGACTGAATTTATTACTATCGTATAC
CCACAAATAAAATAGATTTTAATTAGAGCACTGTTCTAATTAATTAGAGACAAGATTAAGCCAATACTCTCTAATTAAGCT
CCCTAATAAATCAAACTTAATTAGATATTACTTATATTTAACAGATTGTGACTAGATCGCA

TOPO M2/M4 A1 Primer m2/m4 1.0K forward

ATAATAGTCAGTTTATAGTCATCTAAATGTTGAGAGAATTATAATAAAAAATAAAACGTTGGCTATATGATCAGATTTGACCCGAT
TAAAAACCGTATTTATAACGAGCAAGCTTCTATTAAATAGGATATCGTCAAACCTCGCGTTTATAGATTATTACTTAAAACTCAC
CCGAGATTGTGAACAAACAAGATATTATTAGTTTCGCATGCGGAAGCGAGTTCAATGCGGATACGTCACCTTGGCAATGTATCAGT
TTGCTTCCCAAGGATTGTTAAACCTGGCATCAGAGAAACACCCATCGTCACAGTCCCAAGGTCGGTTACCGACTTATGTATGA
ATGCGTCTTTATGACTCGCAACCAACAGCGAAGTGACGATTCCCTCAGCGCTGTACGCGAGCAAGTCCCGCTGGGTCAAAGTA
ATATTGCGGCTCTATTTGCGAGCAACGATACACCTGAATCCAACTGACCTTCACTTACTTACTCTCAGGTCAGCTTATACAAAAC
AGGCTGTGTTTATTTCAACCGGTTGTTATTGTTGCTCAGGATTTATGGCTTTTGCTAAGGTTACAGATAAAGCCCTGGGA
TATCACGCCCTTTAACCAGGCTTAATGAACAATGGGTGGGCAAGTCCAGTTTTTCAGGAACAAAGGTGTCTTAAAGCCAGAAC
TAGAGGCTATTTCTTACAAATACCAATGTGATTGCGTGGCATACATCAGCGATGAGCCCGGCTTTCAGAACTCTCCATCTCTACA
AGA

TOPO M2/M4 A1 Primer m2/m4 1.0K reverse

ACACCTTTGTTTCCCTGAAAACTTGGACCTGCCCAACCCATTGTTTCATTAGCCGGTTAAAGGGCGTGATATCCCCAAGCCTTTTA
TCGTGAACCTTAGCAAAAGCCAATTAATGCCGAGGACAACATAAACAACCCGTTATCAATAACACAGCCGTTTGTGTATAGCT
GACCTGAGAGTAAGTAAGTGAAGTCGATTTGGATTGAGGTGTATCGTTGCTCGCAATAGAGCCGCAATATTACTTTGACCGGAGCG
GGACTTGTCTCGCTGACAGGCGTGAAGGGGAATCGTCACTTCGCTTTTGGTGGTGGAGTCAATAAAGACGCAATTCATCAATTAAGTGG
TAACCGAUCCTTTGGCACTGTGACGATGGGTGTTTCTCTGATGCCAAGTTTAAACAATCCTTGGCGAAGCAAACTGATACATTTCCG
AAGTGAGGTATCCCAATGAACCTCGCTTCCCATGCGAAGCAATTAATATCTTGTGTTGTTCACAATCTCGGGTGAGTTTAAAGTA
ATAACTCTAAACCGGAGTTTACAGATATCTATTTTAATAGAACGTTTCGTCGTTATAAATACGGTTTAAATCGGGTCAAACTCTG
ATCATATAGCCACGTTTATTTTATATATAATCTCTCAACAATTAGATGACTATAACTGACTATATATGAATTAAGTCAC
ATGTTTATTCTGCTGTGTTTTTGGTGGTTCACCAAAAGCATCTCTTCAATTTTATGAAAGAACTCTGCGATCTAGTCACAA
TCTGTAAATATAAGTAATATCTAATTAAGTTTGATTT

TOPO M2/M4 A1.5 Primer m2/m4 1.5K forward

AATGATGGCAAAACAGATCATTGGAACCTTAATATATCAACCTTAAGTGTAAAGGGTCATTGTTGATAAGCAGGTAGGGCGACAACA
AGCTGTGATTCATCGCTTATTTATTTTTCACCGAGAGTAAGTCGATAGTGCCAGCTATGAAAAGAAATAGGGATTGAGAAGGG
CCGGCTCACCTCTAAACCGTAGAACATCGGGTCGGGCACTGTCTATCTTGGACGGACATTAGGTGTAGAACCTCGTTATCGCT
TATTTTGGTGAGTCCATCGGTTGTAATACATTTCCATCTAACCAAGATCTACCCAGCTATGTTGATAACCGTGTCTTCTTGGG
GTGGAGAAGAAGGAATACTCCATATACCAATCAATAACGCTAACAGGGAACAGAGCTACAAATGGCACTATAATTTGGCTCTCT
TTAGGAATAATTCATCTCTTCACTCGCTCGGTCTAATTGGTGGCGGTGTAAGATATTGATGGAGTACGCTGTCTGTAG
AGTATGGAGAGTCTGAAATAGCCGGGCTCATCGCTGATGTATGCCAGCAATCACATTGGTATTTGTAGAGAAATAGCCTCTAGTTC
TGGGCTTAAGGACACCTTTGTTTCCGAAAACTTGGGACCTGCCCAACCCATTGTTTCATTAGCC

TOPO M2/M4 A1.5 Primer m2/m4 1.5K reverse

ATTGTGACTAGCATCGCAGAGTTTCTTTCATAAAAATCAACAATGCTTTTGTGTAACACGCAAAAACACACCAGAAATAAACA
TGTGACTTAAATCATATAGTCAGTTTATAGTCATCTAATTTCTGACAGAATTATAATAAAATAAAACGTTGGCTATATCA
TCAGATTGACCCGATTAATAACCGTATTTATAACGAGCAAGCTTCTATTAATAATAGGATATCGTGAACCTCGGCTTTAGAGTTA
TTACTTAAAAACPCACCCGAGATTGTGAACAACAGATATATTAGTTTTCGCATGGGGAAGCGAGTTTATTTGGGATACGTCACCT
TGCGAAATGATCAGTTTGGTTCGCCAAGGATTTGTTAACTTTGGCCTCAGAGAACACCCATCTGTCAGAGTCCCAAGCTGGGT
ACCGACTTATGATGAATGCGCTTTTATGACTCGCAACCAAGCAAGTACGATTCCCTCAGGCTTCCGCGAGCAAGTGC
CCGCTCGGTCAAAGTAATATTGCGGCTCTATTGCGAGCAACGATACACCTGAATCCAAATCGACTTCACTTACTTACTCTCAGGT
CAGCTTATACAAAACAGGCTGTGTTATTTCAACCGGGTTGTTTAT

Appendix 5

HLV TOPO A+ Primer M13 forward
 AACGGTTTGAGCCTCATCAACATTTCTTTTACGTACACTCTCTTTACCAAAAAACAAATTAACAATACAGCCGTTGAATT
 TAAAAGCTAAATTAATTCACCCCTTTTCAGTATCTATCTATTTATCAACATGAAAAAGTGAAGTTGTCTTGAAGTGCATGGCAC
 AAAAAACATTTATCAAAATAGAAAAATTAACAATGAGGGAAATGTTAATGATCCGAATTAACACAGACTCTCTTAGCAACCGCAGTGT
 TGTCTGTTCTATCTACTGGCGTAAACGCCGAAAAATTTACCCAGACCAAAATAGTCCATGACCAACTTGGTGGCGATGTATGTGCTCT
 GGCTATCGCCCGTTAGACCGCTTTGAAGCCGAAGAGCAAAAGAGTGCTTTGCTGCCTAGCAATGGGCAGTTGGCAGATCACAGGCTT
 AAAAGGAACTGGGTGATCATGGGCCCGGATATAATGGCCTTATTAACAAGATACACCAACGGCCAAAACGTTCTGTATCCGA
 ACAATGACCACTGTGAGATTCCAACTACTCTGCTAAATCAGTTCCAGAGGTAGCGAGTTAGATGTTCAATATGAGTTGGTAAAT
 AACCGTAACGATTTTGTTCGCCCACTAAGCTATCTAGCTCACAACTTGGATA

HLV TOPO A+ Primer M13 reverse
 GGCAATGTGGTTAGCTGGTGCATTGAAGTCCGGCTAAAAACTTGGCTCAAGAATGCGAGTTTATGAAGTGGATGGAAGGCGTGACT
 GGCAATGTGACTAGTGGGGCTAGGCGTTAAGCTTCTAATGGAAGAGCCTGTGTAGTTCACTGTTTCTACTTGAACATGAATATATG
 AAAGCCACGACGCTGTCTGGGCTTTTGTCTTTACGGTGAATCGAGTGTTTTGGCAAAATTAAGTGTAAAGGTGAGTCAATTGTGT
 TATTCAACGACGCTGATGTTTCTATCTCAGCACCTTCGAAACCTAAGTCACTCAACTCATCAGCATTTGAAGTTGGTGGTGACTTCAAT
 ATCGCCAAATGCGTTCACTGCTTTTCTGTTGACGGAATCTGTACTTCTTTTGTACGTAGATTGTTGATAATCGGTGTGCTTGGCCAA
 TCTCAATAGTACCTGCAATTCAGAGTCTGCTAGAGTCAUCCGATACATAAGAGTGAATAGACCTAAGCTTCCGCCAGTGGCG
 TATTGCATGCTTGATAAGCCAGTTCTTTAATAGCCCGAGCCCAATCCACCATTTTGTTTCACTGGAATGTAAAGGTGATCCCA
 TTGGTAGCGAATGTCTGCAGATTATCACTAGCCGCGACCATAGTG

HLV TOPO B+ Primer M13 forward
 AACGGTTTGAGCCTCATCAACATTTCTTTTACGTACACTCTCTTTACCAAAAAACAAATTAACAATACAGCCGTTGAATT
 TAAAAGCTAAATTAATTCACCCCTTTTCAGTATCTATCTATTTATCAACATGAAAAAGTGAAGTTGTCTTGAAGTGCATGGCAC
 AAAAAACATTTATCAAAATAGAAAAATTAACAATGAGGGAAATGTTAATGATCCGAATTAACACAGACTCTCTTAGCAACCGCAGTGT
 TGTCTGTTCTATCTACTGGCGTAAACGCCGAAAAATTTACCCAGACCAAAATAGTCCATGACCAACTTGGTGGCGATGTATGTGCTCT
 GGCTATCGCCCGTTAGACCGCTTTTGAAGCCGAAGAGCAAAAGAGTGCTTTGCTGCCTAGCAATGGGCAGTTGGCAGATCACAGGCTT
 AAAAGGAACTGGGTGATCATGGGCCCGGATATAATGGCCTTATTAACAAGATACACCAACGGCCAAAACGTTCTGTATCCGA
 ACAATGACCACTGTGAGATTCCAACTACTCTGCTAAATCAGTTCCAGAGGTAGCGAGTTAGATGTTCAATATGAGTTGGTAAAT
 AACCGTAACGATTTTGTTCGCCCACTAAGCTATCTAGCTCACAACTTGGATA

HLV TOPO B+ Primer M13 reverse
 GGCAATGTGGTTAGCTGGTGCATTGAAGTCCGGCTAAAAACTTGGCTCAAGAATGCGAGTTTATGAAGTGGATGGAAGGCGTGACT
 GGCAATGTGACTAGTGGGGCTAGGCGTTAAGCTTCTAATGGAAGAGCCTGTGTAGTTCACTGTTTCTACTTGAACATGAATATATG
 AAAGCCACGACGCTGTCTGGGCTTTTGTCTTTACGGTGAATCGAGTGTTTTGGCAAAATTAAGTGTAAAGGTGAGTCAATTGTGT
 TATTCAACGACGCTGATGTTTCTATCTCAGCACCTTCGAAACCTAAGTCACTCAACTCATCAGCATTTGAAGTTGGTGGTGACTTCAAT
 ATCGCCAAATGCGTTCACTGCTTTTCTGTTGACGGAATCTGTACTTCTTTTGTACGTAGATTGTTGATAATCGGTGTGCTTGGCCAA
 TCTCAATAGTACCTGCAATTCAGAGTCTGCTAGAGTCAUCCGATACATAAGAGTGAATGACGCTAAGCTTCCGCCAGTGGCG
 TATTGCATGCTTGATAAGCCAGTTCTTTAATAGCCCGAGCCCAATCCACCATTTTGTTTCACTGGAATGTAAAGGTGATCCCA
 TTGGTAGCGAATGTCTGCAGATTATCACTAGCCG

HLV TOPO A- Primer M13 forward
 AACGGTTTGAGCCTCATCAACATTTCTTTTACGTACACTCTCTTTACCAAAAAACAAATTAACAATACAGCCGTTGAATT
 TAAAAGCTAAATTAATTCACCCCTTTTCAGTATCTATCTATTTATCAACATGAAAAAGTGAAGTTGTCTTGAAGTGCATGGCAC
 AAAAAACATTTATCAAAATAGAAAAATTAACAATGAGGGAAATGTTAATGATCCGAATTAACACAGACTCTCTTAGCAACCGCAGTGT
 TGTCTGTTCTATCTACTGGCGTAAACGCCGAAAAATTTACCCAGACCAAAATAGTCCATGACCAACTTGGTGGCGATGTATGTGCTCT
 GGCTATCGCCCGTTAGACCGCTTTGAAGCCGAAGAGCAAAAGAGTGCTTTGCTGCCTAGCAATGGGCAGTTGGCAGATCACAGGCTT
 AAAAGGAACTGGGTGATCATGGGCCCGGATATAATGGCCTTATTAACAAGATACACCAACGGCCAAAACGTTCTGTATCCGA
 ACAATGACCACTGTGAGATTCCAACTACTCTGCTAAATCAGTTCCAGAGGTAGCGAGTTAGATGTTCAATATGAGTTGGTAAAT
 AACCGTAACGATTTTGTTCGCCCACTAAGCTATCTAGCTCACAACTTGGATA

HLV TOPO A- Primer M13 reverse
 GGCAATGTGGTTAGCTGGTGCATTGAAGTCCGGCTAAAAACTTGGCTCAAGAATGCGAGTTTATGAAGTGGATGGAAGGCGTGACT
 GGCAATGTGACTAGTGGGGCTAGGCGTTAAGCTTCTAATGGAAGAGCCTGTGTAGTTCACTGTTTCTACTTGAACATGAATATATG
 AAAGCCACGACGCTGTCTGGGCTTTTGTCTTTACGGTGAATCGAGTGTTTTGGCAAAATTAAGTGTAAAGGTGAGTCAATTGTGT
 TATTCAACGACGCTGATGTTTCTATCTCAGCACCTTCGAAACCTAAGTCACTCAACTCATCAGCATTTGAAGTTGGTGGTGACTTCAAT
 ATCGCCAAATGCGTTCACTGCTTTTCTGTTGACGGAATCTGTACTTCTTTTGTACGTAGATTGTTGATAATCGGTGTGCTTGGCCAA
 TCTCAATAGTACCTGCAATTCAGAGTCTGCTAGAGTCAUCCGATACATAAGAGTGAATGACGCTAAGCTTCCGCCAGTGGCG
 TATTGCATGCTTGATAAGCCAGTTCTTTAATAGCCCGAGCCCAATCCACCATTTTGTTTCACTGGAATGTAAAGGTGATCCCA
 ATTGGGTAGCGAATGTCTGCAGATTATCACTAGCCG

HLV TOPO B- Primer M13 forward
 AACGGTTTGAGCCTCATCAACATTTCTTTTACGTACACTCTCTTTACCAAAAAACAAATTAACAATACAGCCGTTGAATT
 TAAAAGCTAAATTAATTCACCCCTTTTCAGTATCTATCTATTTATCAACATGAAAAAGTGAAGTTGTCTTGAAGTGCATGGCAC
 AAAAAACATTTATCAAAATAGAAAAATTAACAATGAGGGAAATGTTAATGATCCGAATTAACACAGACTCTCTTAGCAACCGCAGTGT
 TGTCTGTTCTATCTACTGGCGTAAACGCCGAAAAATTTACCCAGACCAAAATAGTCCATGACCAACTTGGTGGCGATGTATGTGCTCT
 GGCTATCGCCCGTTAGACCGCTTTTGAAGCCGAAGAGCAAAAGAGTGCTTTGCTGCCTAGCAATGGGCAGTTGGCAGATCACAGGCTT
 AAAAGGAACTGGGTGATCATGGGCCCGGATATAATGGCCTTATTAACAAGATACACCAACGGCCAAAACGTTCTGTATCCGA
 ACAATGACCACTGTGAGATTCCAACTACTCTGCTAAATCAGTTCCAGAGGTAGCGAGTTAGATGTTCAATATGAGTTGGTAAAT
 AACCGTAACGATTTTGTTCGCCCACTAAGCTATCTAGCTCACAACTTGGATA

HLV TOPO B- Primer M13 reverse
 GCAATGTGGTTAGCTGGTGCATTGAAGTCCGGCTAAAAACTTGGCTCAAGAATGCGAGTTTATGAAGTGGATGGAAGGCGTGACTG
 GCAATGTGACTAGTGGGGCTAGGCGTTAAGCTTCTAATGGAAGAGCCTGTGTAGTTCACTGTTTCTACTTGAACATGAATATATG
 AAAGCCACGACGCTGTCTGGGCTTTTGTCTTTACGGTGAATCGAGTGTTTTGGCAAAATTAAGTGTAAAGGTGAGTCAATTGTGT
 TATTCAACGACGCTGATGTTTCTATCTCAGCACCTTCGAAACCTAAGTCACTCAACTCATCAGCATTTGAAGTTGGTGGTGACTTCAAT
 TCGCCAAATGCGTTCACTGCTTTTCTGTTGACGGAATCTGTACTTCTTTTGTACGTAGATTGTTGATAATCGGTGTGCTTGGCCAA

CTCAATACTAGCTCCAAATTGAGAGTCTGCCGTAGAAGTCACCCGATACATAAGAGTGGAAATGACGTAAGCTTCCGCCAGTGGCGT
ATTGCATGCTTGATAAGCCAGCTTCTTTAATAGCCCCAGCCCCAATCCCACCATTTTGTTCACCTGGAATGTAACGGTCAATCCCAT
TGCTAGCGAATGTCTGCAGATTATCACTCCCC

Appendix 6

Full DMC-1-M2/M4 sequence 8684 bp

aagtcattta	ataggcaaca	agtaacacat	catgttggtta	tacgtaacta	ttcacaaaata	60
tctaagtaaa	caatgaaaac	acttaaatca	tcaatagtga	gaaagaatcg	gctctacgct	120
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agagttggca	ttatcttgtt	tctcacacgc	gtcagatttc	gcaccccatg	atgtgccatc	360
taagaacaca	tcacacgctt	tcttaagtga	accaaggttc	tgttcaagct	cgtctcttcc	420
ttttctcaagc	ttttcaagtt	cttgagatat	ttgtaattct	ttacggaaca	gttcacgaga	480
acgttcaaac	aaggtcgact	gcataaccgc	ctgacggttt	agcttttgat	tatcttgctc	540
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tgcttccaga	cgtgcgtatc	acaactcgct	acgatgaagc	t		8681

Note: The open reading frames (orfs) with significant matches are highlighted in bold text as follows:
 Sodium/alanine symporter 1812-3017 bp, ToxR (blue) 3574-4083 bp, Splendylisin (red) 5931-6869 bp,
 Hypothetical protein 6993-7505 bp, Thermostable carboxypeptidase 7935-8684 bp.

Appendix 7**Splendilysin nucleotide and amino acid sequence**

atgatccgaataaacagaagtctcttagcaaacgcagtggtgtctgttctatctactggc
 M I R I N R S L L A T A V L S V L S T G
 gtaaacgcgaaaaatttaccagaccaaatagtccatgaccaeacttggtgacgatgtatgt
 V N A K I Y P D Q I V H D Q L G D D V C
 cgctctgggtatcgccggttagacogctttgaagccgaagagcanaagagtgcttugctg
 R S G Y R P L D R F E A E R Q K S A L L
 qctagaatgggcacttggcagatcacaggcttataaaggaaaactgggtgatcatggggcccg
 A R M G T W Q I T G L K G N W V I M G P
 ggatataatggccttattaaacaagatacaaccaacggcaaaaacggttctgttatccgaac
 G Y N G L I K Q D T T N G K T F C Y P N
 aatgaccagtgctgagattccaaactactctgctaaatcagttccagaaggtagcgagtta
 N D Q S E I P N Y S A K S V P E G S E L
 gangttcaatatgagttggtaataaacogtaacgattttgttcgcccactaagctatcta
 D V Q Y E L V N N R N D F V R P L S Y L
 gctcacaaccltggatacggctgggttggcggttaataacagtcagtcagttggcgaagac
 A H N L G Y A W V G G N N S Q Y V G E D
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 M T I K R S G D S W V I Q G N N G G S C
 agcgggttatcgctgtgggtgaaaaaacnaaaatcacogtcgacaactttgcctacacogtt
 S G Y R C G E K T K I T V D N F A Y T V
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 N D D N F W H G D V V E S D R E L V K T
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 A N Q S F S K S N G G S T S E Q V T L Q
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 A R P M V P A N S E L P I R V E L Y R S
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 S I S Y P Y R F N A D I S Y D V E F N G
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 F L R W G G N A W H T H P D N R P Y R A
 cacaccttcactatgggtcgcggcagtgataaatctgcagacattcgctaccaatgggat
 H T F T M G R G S D K S A D I R Y Q W D
 caccggttacattccaggtgaaacaaaatgggtgggattggggctgggctatlaaagaagct
 H R Y I P G E T K W W D W G W A I K E A
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 G L S S M Q Y A T G G S L R P F H S Y V
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 S G D F Y A D S Q F A G T I E I G Q A T
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 P I I N N L R S K R S T D S V N E T T E
 cgcattggcgatattgaagtcaccaccaacttcaatgctgatgaglttagtgacttaggt
 R I G D I E V T T N F N A D E L S D L G
 ttccgaaggtgctgagatgaacatcagcgctcgttgaataa
 F E G A E M N I S V V E -

Appendix 8 Protocols

SDS-PAGE NuPAGE Bis-Tris Gels

Protein samples were dispensed into eppendorf tubes (62 μ l) with 3 μ l of ultrapure water, 25 μ l 4X NuPAGE LDS sample buffer (see Appendix 9) and 10 μ l of reducing agent DTT (Dithiotheritol). Samples were heated at 70 °C for 10 min. The 20X NuPAGE SDS Running Buffer (MOPS-Appendix 9) was diluted to 1X in ultrapure water (up to 1L). An 800 ml volume was set aside for the lower chamber and 500 μ l of NuPage antioxidant was added to the remaining 200 ml for the upper chamber. Precast 12 % Bis-Tris gels were removed from packaging, comb removed and rinsed thoroughly with 1X SDS running buffer prior to use. Gels were locked into the Mini-Cell and upper buffer was added to the chamber. Ten μ l volumes of sample were loaded into the wells. The lower chamber was filled with 1X lower running buffer and electrophoresis was at 200 V (constant) for 50 min.

QIAprep Miniprep Kit protocol

Overnight cultures (5 ml) of *E. coli* containing plasmid grown at 37 °C in LB medium were harvested at 4,000 rpm for 10 min. Pelleted bacterial cells were resuspended in 250 μ l of Buffer P1 and transferred to microfuge tubes. Buffer P2 (250 μ l) was added and tubes were gently inverted to mix, 350 μ l of Buffer N3 was added and tubes were inverted immediately. Samples were centrifuged at 13,000 rpm x 10 min in bench top microcentrifuge. Supernatants were transferred directly into QIAprep spin columns by decanting solutions. Columns were centrifuged for 1 min, flow-through discarded, columns washed with 750 μ l Buffer PE and centrifuged for a further 1 min. Flow-through was discarded and columns were centrifuged for an additional 1 min to remove residual wash buffer. Columns were transferred into clean 1.5 ml microfuge tubes and plasmid DNA was eluted with 50 μ l Buffer EB (10 mM TrisHCl, pH8.5) by addition to the centre of column, standing for 1 min and centrifugation for 1 min.

Addition of 3' A-Overhangs Post-amplification

After amplification with proofreading polymerase, vials were placed on ice and 0.7-1 unit of *Taq* polymerase was added to each tube, mixed well and incubated at 72 °C for 10 min. Samples were held on ice and used immediately in the TOPO Cloning reaction.

TOPO Cloning Reaction

A 0.5-4 μ l volume of PCR product was added to 1 μ l salt solution, 1 μ l TOPO vector and made up to 6 μ l with sterile water. The reaction was mixed gently and incubated at room temperature for 5 min. The reaction was placed on ice whilst competent cells were prepared. One vial of One Shot chemically competent TOP10 cells were thawed on ice for each transformation. A 2 μ l volume of the TOPO Cloning reaction was added to the competent cells, mixed gently and were held on ice for 5 to 30 min. Cells were heat-shocked at 42 °C for 30 sec and were immediately transferred to ice. SOC medium (250 μ l) was added to each vial and incubated at 37 °C for 1 h with agitation. A 10-50 μ l volume of each transformation was spread onto prewarmed selective plates (LB agar containing 50 μ g ml⁻¹ ampicillin) that were incubated overnight at 37 °C.

Appendix 9 Bacterial culture media and buffers**Marine Agar (MA)**

Difco Marine Agar 2216 55.1 g/L
Boiled and autoclaved at 121 °C for 15 minutes.

Marine Broth (MB)

Difco Marine Broth 2216 37.4 g/L
Autoclaved at 121 °C for 15 minutes.

TCBS

TCBS agar 88 g/L
Boil to dissolve.

Hugh and Leifson Medium

Hugh and Leifson (1953) medium was prepared with 1.5 % NaCl and autoclaved at 121 °C for 15 min. To this medium 10 % filter-sterilised D-glucose solution was added. The tubes were prepared in duplicate, one set with liquid paraffin and the other without.

DNase test agar

DNase agar 42 g/L
1 % NaCl
Autoclave at 121 °C for 15 min. Add 0.02 g of Bromophenol blue suspended in dH₂O.

Luria-Bertani (LB) broth

Tryptone 10 g/L
NaCl 10 g/L
Technical Yeast Extract 5 g/L
Add 1.5 % technical agar to make LB agar.
Autoclave at 121 ° for 15 min

f/2 medium

f/2 Guillard's marine water enrichment solution (sterile) 20 ml/L
Autoclaved Seawater 980 ml/L

SOC Medium

Tryptone 20 g/L
Yeast extract 5 g/L
NaCl 0.5 g/L
KCl (250 mM) 10 ml
pH 7.0
Autoclave at 121 °C for 15 min. Add 5 ml of 2 M MgCl₂ and 20 ml 1 M glucose.

NZY Agar

NaCl 5 g/L
MgSO₄ · 7H₂O 2 g/L
Yeast Extract 5 g/L
NZ amine 10 g/L
Technical agar 15 g/L

Adjust pH to 7.5 with NaOH
Autoclave at 121 ° for 15 min

NZY Broth

NaCl	5 g/L
MgSO ₄ · 7H ₂ O	2 g/L
Yeast Extract	5 g/L
NZ amine	10 g/L
Adjust pH to 7.5 with NaOH	
Autoclave at 121 °C for 15 min.	

NZY Top Agar

1 L of NZY broth
Add 0.7 % (w/v) agarose
Autoclave 121°C for 15 min.

2YT Broth

Tryptone	16 g/L
Yeast Extract	10 g/L
NaCl	5 g/L
pH 7.0	
Autoclave at 121 ° for 15 min	

SM Buffer

NaCl	5.8 g/L
MgSO ₄ · 7H ₂ O	2.0 g/L
1M Tris-HCl	50.0 ml/L
2 % Gelatin (w/v)	5.0 ml/L
Autoclave at 121°C for 15 min.	

Nine Salt solution (NSS)

NaCl	17.6g/L
Na ₂ SO ₄	1.47 g/L
NaHCO ₃	0.08 g/L
KCl	0.25 g/L
KBr	0.04 g/L
MgCl ₂ ·6H ₂ O	1.87 g/L
CaCl ₂ ·2H ₂ O	0.41 g/L
SrCl ₂ ·6H ₂ O	0.008 g/L
H ₃ BO ₃	0.008 g/L

Solution was made up in 5 mM MgSO₄ solution.

Bis-Tris buffer

20 mM Bis-Tris
0.9 % NaCl
pH 6.8

SDS-PAGE solutions**NuPAGE LDS Sample Buffer 4X (10 ml)**

Glycerol	4.0 g
Tris Base	0.682 g

Tris HCl	0.666 g
Lithium dodecylsulphate (LDS)	0.800 g
EDTA	0.006 g
Serva Blue G250	0.75 ml of 1 % solution
Phenol Red	0.25 ml of 1 % solution
Ultrapure water to 10 ml	
1X buffer should be pH 8.5	

Running buffer**MOPS SDS Running Buffer (20X) 500 ml**

MOPS 3-(-N-morpholino) ethane sulfonic acid	104.6 g (1 M)
Tris base	60.6 g (1 M)
SDS	10.0 g
EDTA	3.0 g
Ultrapure water to 500 ml.	
1X buffer should be pH 7.7	

Stain solution

Coomassie Brilliant Blue R250	1.25 g/500 ml
50 % Methanol	454 ml
Glacial acetic acid	46 ml

Fixative

50 % Methanol
10 % Acetic acid
40 % dH ₂ O

Destain

30 % Methanol
10 % Acetic acid
60 % dH ₂ O

Electrophoretic transfer to PVDF membrane**Transfer buffer**

Bicine (25 mM)	4.08 g/L
Bis-Tris (25 mM)	5.23 g/L
EDTA	0.29 g/L
Methanol	100 ml/L
Ultrapure water (MilliQ)	Make up to 1 litre

Blocking solution

Marvel	1.5 g
PBS	50 ml

DNA electrophoresis**TE Buffer**

Tris-HCl	10 mM
EDTA	1 mM

Appendix 10

Data taken from Honours Microbiology Project (Sheriff, 2004).

Glucose addition to MB and TSB + 1.5 % NaCl media

A stock solution of glucose was added to both MB and TSB + 1.5 % NaCl to final concentrations of 0.5% and 1%. Broths were inoculated with single colonies grown on MA and TSA + 1.5 % NaCl for 24 h, respectively. Cultures were grown for 2 days and were sampled and analysed for haemolysin production, numbers of viable bacterial cells at 7.5 hrs, 25 hrs, and 31 hrs were measured to determine if the addition of glucose had any effect on growth of the organism and its ability to produce toxin. The cultures with added glucose were set up with controls with no added glucose in the same media. Glucose was added to on-growing cultures of TSB + 1.5 % NaCl at 24 hrs when toxin production was thought to begin. It was shown that the addition of glucose slightly inhibited the growth of *V.splendidus*, which was more evident in TSB + 1.5 % NaCl (Sheriff, 2004). Although the growth of the organism was only slightly affected by increased glucose levels, haemolysin production was severely inhibited with no toxin production observed for cultures with added glucose at any time period sampled (Figure 1).

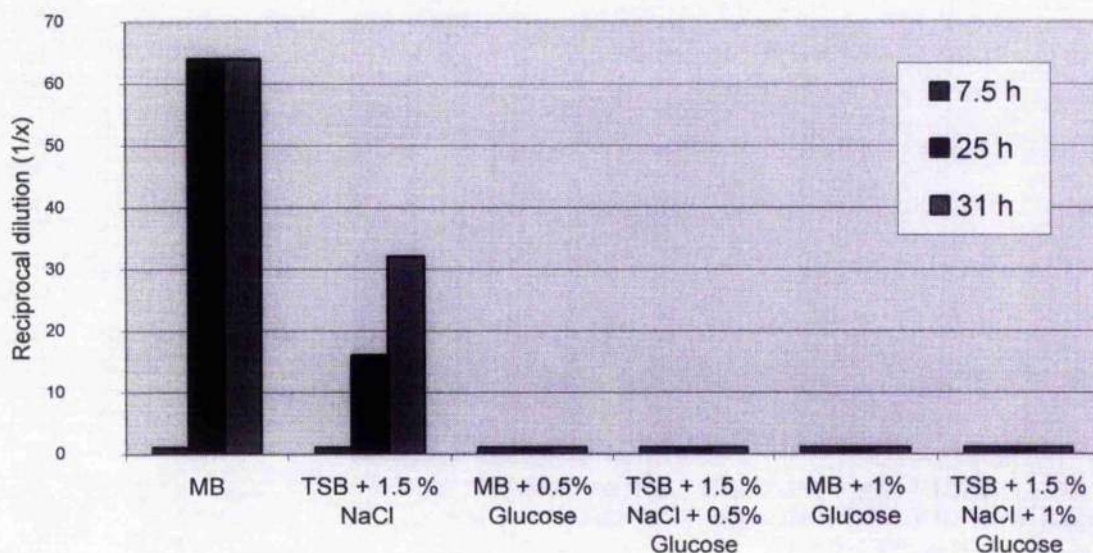


Figure 1. Haemolysin production in MB and TSB + 1.5 % NaCl with and without the addition of glucose over a growth period of 31 hours. Glucose was added at a final concentration of 0.5 and 1 %.

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The Ropewalk, Schofield Street,
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